Characterization of Isolated Autologous-Blood Perfused Rat Heart Model: Validation and Applications for Investigation of Acute Hypoxic Responses in Rat Heart

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C. Abbreviations

3R principles	Reduce, Refine, Replace
aVL	Lead augmented vector left projection
ECG	Electrocardiogram
GSH	Reduced glutathione
GSSH	Oxidized glutathione
Hb	Haemoglobin
Hct	Haematocrit
HR	Heart rate
hrEpo	Human recombinant erythropoietin
L-NAME	N^G- Nitro-L-arginine methyl ester
LV	Left ventricle
LVP	Left ventricular pressure
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
RV	Right ventricle
SERCAs	Sarco/ endoplasmic reticulum Ca^{2+} ATPases

1. Summary

The isolated perfused heart model developed by Langendorff allows the monitoring of a broad spectrum of functional, biochemical, morphological and pathological indices in a denervated heart under controlled microenvironmental conditions. Main limitations of this method include the lack of mechanical loading of the heart muscle and poor oxygen supply due to the low oxygen carrying capacity of perfusion fluids without red blood cells. As a result rodent hearts perfused via aorta with the Krebs-Henseleit buffer are usually artificially paced.

Herein I introduced a novel hollow fibre mini-oxygenator into the Langendorff perfusion circuit allowing to reduce its filling volume from 100 ml to 5 ml and less. Due to this technical improvement 5-10 ml of blood obtained from the donor rat used for heart harvesting are sufficient to fill the system and the isolated heart can therefore be perfused with autologous blood.

This study was focused on the characterization of a new experimental model and the comparison of functional and biochemical parameters obtained for the autologous blood perfused heart with those measured *in vivo*. Potential limitations of this method were specifically addressed.

Furthermore, we performed a study in which the new system was used to investigate local responses of the isolated heart to hypoxia and ischemia. We assessed changes in glucose utilization as well as the associated changes in the tissue redox state and ion balance. The obtained data revealed inter-ventricular heterogeneity in responses of the rat heart to hypoxia that are most likely linked to the heterogeneity in the functional requirements.

2. Zusammenfassung

Das von Langendorff entwickelte, isolierte, perfundierte Herzmodell ermöglicht die Beobachtung eines breiten Spektrums funktioneller, biochemischer, morphologischer und pathologischer Parameter im denervierten Herzen unter kontrollierten Mikroumgebungsbedingungen. Die wichtigsten Beschränkungen dieses Verfahrens sind die fehlende mechanische Belastung des Herzmuskels und der mangelnde Sauerstofftransport aufgrund der geringen Sauerstoffbindungskapazität der erythrozytenfreien Perfusionsflüssigkeiten. Deshalb werden Nagetierherzen, die über die Aorta mit dem Krebs-Henseleit Puffer perfundiert werden, in der Regel per externem Schrittmacher stimuliert.

In dieser Arbeit integriere ich einen neuartigen Hohlfaser-Mini-Oxygenator in den Langendorff-Perfusionskreislauf und verringere so die benötigte Füllmenge von 100 ml auf 5 ml oder weniger. Dank dieser technischen Verbesserung genügen 5–10 ml Blut von der Spenderratte, von der auch das Herz stammt, um das System zu füllen. Das isolierte Herz kann also mit Eigenblut perfundiert werden.

Im Mittelpunkt dieser Studie stehen die Charakterisierung eines neuen experimentellen Modells und der Vergleich funktioneller und biochemischer Parameter des mit Eigenblut perfundierten Herzens mit den Werten *in vivo*. Auf potenzielle Beschränkungen der Methode wird spezifisch eingegangen.

Darüber hinaus wurde das neue System in einer Studie eingesetzt, in der lokale Reaktionen des isolierten Herzens auf Hypoxie untersucht wurden. Gemessen wurden Veränderungen des Glukoseverbrauchs sowie die damit verbundenen Veränderungen im Redoxzustand und im Ionengleichgewicht des Gewebes. Die gesammelten Daten zeigten, dass die interventrikuläre Heterogenität in der Reaktion des Rattenherzens auf Hypoxie mit höchster Wahrscheinlichkeit auf die Heterogenität der funktionalen Anforderungen zurückzuführen ist.

3. Motivation and structure of the thesis work

The present study aimed to characterize isolated rat heart responses to acute global hypoxia and to compare the observed findings with those obtained for the heart of animals exposed to systemic hypoxia *in vivo*. Moreover, we planned to assess possible interventricular variation in hypoxic responses in rat hearts.

The comparison of the data obtained *ex vivo* and *in vivo* allowed us to study the acute effects of hypoxic stress in the rat heart in the presence and absence of sympathetic and vagal stimulation, assess the possible impact of loading on the observed hypoxic responses, and the presence of humoral factors released systemically in response to hypoxic exposure.

This PhD thesis is structured as follows. In an introduction, a brief overview of the methods used in cardiovascular research is given, explaining the motivation for the development of a new experimental and technical approach which allows avoidance of some artifacts related to the pre-existing experimental settings. After the introductions follows the body of the thesis which describes the validation of the new experimental model and presents the data obtained in a test study on the acute responses to hypoxia of the left and right ventricles of the rat heart.

In the introduction, which is subdivided into two parts, some of the most important tools for assessing the heart physiology are summarized. Further, features of the validation of a new model are cited, the isolated autologous blood-perfused rodent heart, which was crucial for the performance of the target study. Technical characteristics of this method, namely the autologous blood rodent heart perfusion, were described and discussed in this chapter. Animal handling, heart and blood harvesting and initiation of the perfusion system as well as the necessary troubleshooting are also described here.

Section 5 describes the assessment of the local changes in tissue ion content, redox state and glucose metabolism in response to acute hypoxia. The data obtained from the hearts harvested from rats exposed to systemic normobaric hypoxia for one hour were compared to those obtained in the isolated rat hearts perfused with hypoxic autologous blood. Special attention was paid to the right-to-left ventricular heterogeneity in hypoxic responses in both experimental settings. The use of isolated autologous blood-perfused rodent hearts provided an opportunity to precisely control the amount of oxygen delivered to the organ and to distinguish between the systemic

and autonomous responses of the heart to hypoxia. The findings of that study are summarized in section 6. These include the assessment of interventricular heterogeneity in the glucose utilization rate in normoxic and hypoxic hearts, as well as the capability for maintaining tissue ATP levels and redox state under conditions of reduced oxygen supply. The interventricular Na^+ gradients that were maintained in the heart regardless of oxygen availability were monitored in both *in vivo* and *ex vivo* settings. These differences could be at least partially explained by the variations in the Na/K-ATPase activity between the right and the left ventricles. Taken together the observed differences in glucose metabolism, antioxidative defense systems and ion handling, these most likely reflect the interventricular differences in force generation.

The present study revealed certain limitations of our model, one of which is a lack of loading. Data on redox state responses to hypoxia generated using an *ex vivo* model differed from those obtained *in vivo*. However, the changes in ion content and in glucose utilization rate correlated well between the *in vivo* and *ex vivo* models.

The results obtained allowed us to follow the correlation between the local glucose utilization, the maintenance of ion gradients and the redox state. These observations indicate that anaerobic glycolysis is an essential element required to preserve the myocardial tissue from necrosis and sustain contractile function in hypoxic myocardium. Furthermore, we suggest that the RV is more susceptible to hypoxia-induced damage than the LV.

4. Introduction

Efficient prevention and treatment of cardiovascular diseases is based on the understanding of pathophysiological mechanisms underlying those disorders. Despite impressive results obtained for the therapeutic interventions using all available experimental models from *in vivo* animal studies in organ and cellular models, progress in the actual treatment of patients with cardiovascular diseases is modest (Mathers *et al.*, 2009). Therefore, there is a long-standing demand for novel experimental tools and approaches to improve the outcome of cardiovascular research. Ideal experimental models must adequately reflect the clinical manifestation of pathology, as well be easy to handle and standardize. The current chapter introduces the variety of experimental models currently in use in cardiovascular research. Special attention is given to the Langendorff isolated perfused heart model.

This section summarizes the results obtained during validation of the newly introduced blood-perfused heart model. These findings will be compared with the ones generated with the classical Langendorff model and the results of *in vivo* studies. The advantages and limitations of the newly developed model will be discussed.

Exploration of the experimental potential of the model will be exemplified in the experiments which used the improved method to study effects of acute hypoxia in the denervated heart. These studies are summarized in chapter 5 and 6 of the thesis.

4.0 Experimental models in cardiovascular clinical research

At present, a broad array of experimental models from single molecules and molecular complexes to freshly isolated heart cells, isolated organs as well as small (rodents) and large animals models (Table 1) are used for the following purposes: a) characterisation of the molecular mechanisms of diseases; b) drug acute toxicity testing; c) development of new therapeutic strategies including *i)* optimisation of drug administration (acute and long-term studies), *ii)* drug turnover and metabolism, *iii)* optimization and development of new surgical procedures and *iv)* testing of the surgical equipment and consumables; and d) patient handling during and after the

invasive treatment phase. Each of these models has its own advantages and limitations which are summarised in Table 1 below.

Table 1: Models and their applications

Models	Molecular mechanism of disease	Functional performance of diseased heart	Drug toxicity	Drug metabolism and turnover	Drug administration	Surgical procedures, development and testing new surgical equipment and	Patient management
Molecular models	+	-	-	-	-	-	-
Cells	+	±	-	-	-	-	-
Strips	+	+	±	±	±	-	-
ex vivo saline perfused heart	+	+	+	±	±	±	-
ex vivo blood perfused heart	+	+	+	+	+	±	±
in vivo models	+	+	+👎	+👎	+👎	+👎	±

Legend:
+ Suitable;
± Difficulties in interpretation/poor clinical relevance;
- Not suitable;
👎 Incompatible with 3R principle (Remove, Reduce, Refine)

4.1 Skinned fibres

The heart ventricles are characterised by substantial electrical and mechanical heterogeneity (Markhasin *et al.*, 2003). Therefore, an efficient heart function is dependent on the precise combination of electrical activation and myocardium activation (Markhasin *et al.*, 2003). Several experiments which assess mechanical properties of the cardiac muscle are performed in skinned fibre. Chemical skinning results in intact contractile machinery and Ca^{2+} concentration can be precisely controlled through the use of Ca^{2+}/EGTA buffers. Experiments based on measurement of Ca^{2+} tension transients followed by experiments in calcium step

release and length step can be simplified in a mathematical model that considers (i) passive elasticity of cardiac tissue; (ii) Ca-binding to troponin C and its dependence on crossbridge tension; (iii) thin filament kinetics and associated length dependence; and (iv) crossbridge kinetics (Hunter *et al.*, 1998).

4.2 Cells

Enzymatic digestion of the myocardial tissue has been developed to obtain adult or neonatal cardiomyocyte preparations. Among the main cell cultures used are i) neonatal cardiomyocytes (may be maintained in a culture for several days) (Webster & Patrick, 2000); ii) fresh-isolated adult cardiomyocyte (may be used during the first hours after isolation before the remodelling process is initiated) (Severs *et al.*, 1989); and iii) cells lines such as HL-1 (Claycomb *et al.*, 1998) and MCM1 (Sculptoreanu *et al.*, 1992).

Among the parameters assessed using isolated cardiomyocytes are electrophysiological recordings, Ca^{2+} handling, signalling mechanisms and role of the proteins of interest, as well as force development and function of the contractile machinery in paced cells. Among the experimental techniques requiring isolated cardiomyocytes are various forms of live imaging, patch clamping, force detection, western blot and immunohistochemistry as well as PCR, transfection and gene silencing. Lack of the intercellular contacts and cross-talk between different cell types, as well as loading and pressure distribution are among the limitations of this experimental model (Diaz & Wilson, 2006).

4.3 Myocardial tissue strips

Myocardial tissue strips can provide important information regarding the length-dependent sensitivity of the contractile system to Ca^{2+}. This type of preparation is a powerful tool for the investigation of effects of filament geometry on maximal force development and the effect of stretch of uniform muscle on sensitivity of the contractile system for Ca^{2+} ions (ter Keurs *et al.*, 2008). This preparation is limited to mimicking the intact organ geometry (Klocke *et al.*, 2007).

4.4 Ex vivo heart perfused models

4.4.1 Isolated heart models

The isolated retrograde perfused mammalian heart first described by Oscar Langendorff (Langendorff, 1898) is a simple method which allows the measurement of a broad spectrum of biochemical, physiological, morphological and pharmacologic indices in the mammalian myocardium (Sutherland & Hearse, 2000). Furthermore, the isolated organ is not subjected to sympathetic and vagal stimulation, enabling the monitoring of responses of the myocardium itself to the experimental stimuli. This low-cost and easy-to-manage model is often a major choice for the adjustment of doses of medicaments and the duration of treatments prior to the *in vivo* testing experiments. One of the main limitations of this method is the absence of blood or blood components in perfusion fluid, resulting in reduced oxygen supply to the myocardial tissue.

4.4.2 Isolated perfused mammalian heart: a model of choice over 100 years

Since its inception, the perfused mammalian heart preparation model has undergone some modifications but its basic principles remain unchanged (Fig 1). It allows simulation of low-flow to no-flow local or global ischemia, loss of rhythmicity and other pathologic conditions. The Langendorff model has been successfully used to study the molecular and cellular mechanisms underlying cardiovascular disorders, and for the development of acute therapeutic interventions and donor organ preservation strategies. (de Leiris *et al.*, 1984; Curtis, 1998; Valentin *et al.*, 2004; Skrzypiec-Spring *et al.*, 2007)

4.4.3 Perfusion fluids used in the Langendorff model

The majority of scientists using the Langendorff heart perfusion system fill the perfusion circuit with the Krebs-Henseleit buffer containing: 25.0 mM $NaHCO_3$ (2.1 g/L), 118 mM NaCl (6.9 g/L), 4.7 mM KCl (0.35 g/L), 1.2 mM $MgSO_4$ (anhydrous) (0.145 g/L), 1.2 mM NaH_2PO_4 (0.145 g/L), 1.2 mM $CaCl_2$ (0.175 g/L), optionally containing 2 g/L glucose. The buffer is equilibrated with gaseous phase containing 5% CO_2, 95% O_2 and warmed to 37°C.

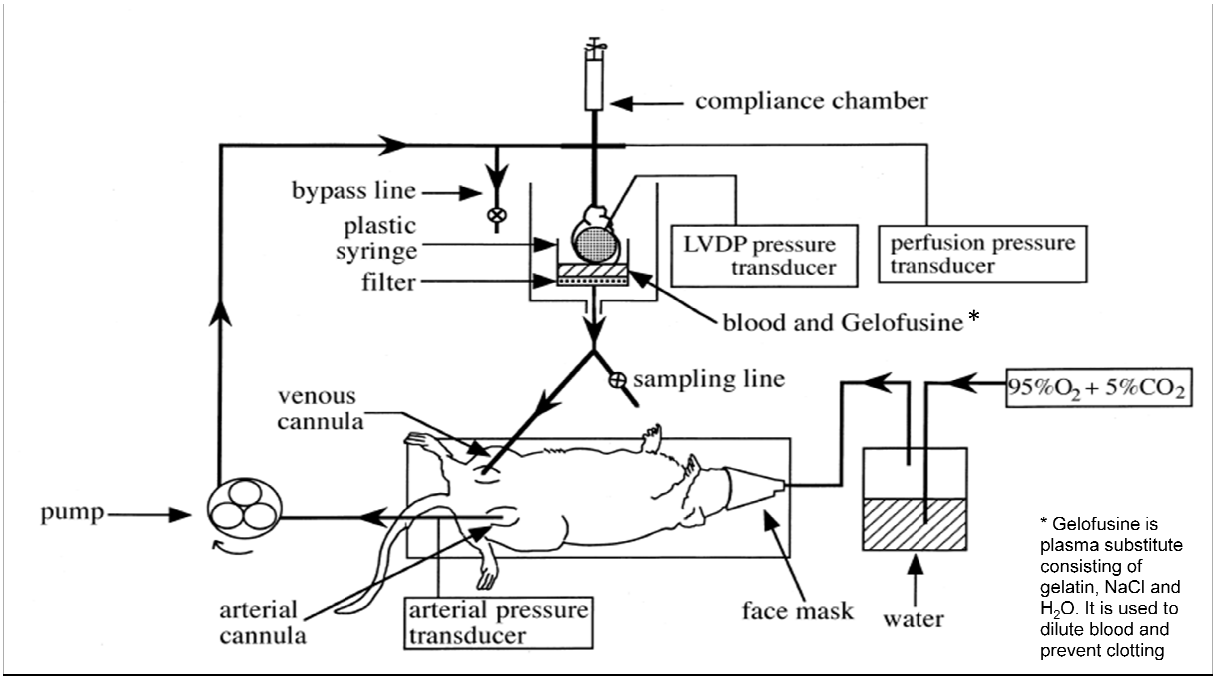

Figure 1: Blood-perfused myocardium with a support animal setting (from Sutherland and Hearse, 2000).

This buffer was developed based on the ionic composition and pH of blood plasma. However, it lacks plasma proteins, amino acids and other nutrients including fatty acids. Its viscosity and the form in which ions are present in the liquid phase differs substantially from that in plasma (Sutherland & Hearse, 2000; Skrzypiec-Spring *et al.*, 2007). Furthermore, due to the absence of red blood cells, the oxygen carrying capacity of Krebs-Henseleit buffer is ~50-fold lower than that of blood even after equilibration with 95% O_2-containing gaseous phase. As a result, oxygen availability is limited and the spontaneous heart rate as well as the contractile force is significantly reduced when compared to those *in vivo* (Sutherland & Hearse, 2000; Skrzypiec-Spring *et al.*, 2007). Solutions have been proposed to improve O_2 availability to the myocardium and included perfusion with washed erythrocytes collected from a different species (goat or cow), perfusion with blood collected from several additional animals of the same species, or using a support animal as a natural heart-lung machine (Fig 1).

Use of whole blood or erythrocytes of a different species poses a number of problems such as inter-species incompatibility, release of substances with unknown effects, and differences in erythrocyte size and viscosity. Blood collection from rodents of the same species may solve the problem, but the number of “blood

donors" to be used in a single experiment will be unforgivably high (5 rats or 67 mice). This increases experimental costs and does not comply with the 3R principle of animal experimentation.

Using parabiotic preparations with a support animal (Fig 1) can reduce some of those complications. However, this model is almost as complex as *in vivo* models and does not allow precise control over the doses of applied drugs (they are often metabolized by the support animal). In addition, side and secondary effects of the support animal to the administrated drugs cannot be excluded or assessed reliably. It is difficult to control the blood oxygenation as it depends on the respiratory pattern of the support animal remaining under anaesthesia. Blood trauma will slowly occur over time as erythrocytes will be damaged when passing through the peristaltic pump, and the increase in plasma K^+ will gradually impact heart function (Sutherland & Hearse, 2000).

Yet one more alternative approach is based on the development of haemoglobin-free blood substitutes whose oxygen carrying capacity exceeds that of the Krebs-Henseleit buffer. Among those are perfluorochemical oxygen-carrying haemoglobin substitutes which are capable of transporting 5-10 ml O_2/100 ml fluid (Lowe, 1999). Despite being efficient oxygen carriers, such O_2 binding agents are unable to mediate O_2 delivery and release of O_2 in hypoxic tissues as effectively as intraerythrocytic haemoglobin (Table 2).

Table 2: Oxygen carrying capacity and efficiency of oxygen delivery for different perfusion fluids

	O_2 carrying capacity (in 100ml fluid at 37°C)	**O_2 delivery capacity**	**Side effects**
Krebs-Henseleit buffer	~0.4 ml O_2	low	None
Haemoglobin-free blood substitutes	5-10 ml O_2	low-medium	Vasoconstriction
Blood	20 ml O_2	high	None

4.5 *In vivo* models

In vivo models are most reliable in reflecting the actual development of cardiovascular diseases. Rodents (mice and rats) and larger mammals (pigs and sheep) are extensively used for each of the above-mentioned model applications in Table 1. Existence of numerous transgenic mouse lines allows identification of the underlying mechanisms at the molecular level and selection of the potential drug targets. Rats are extensively used to investigate the causes and consequences of hypertrophy, myocardial infarction, cold global ischaemia-reperfusion and rejection (heterotrophic heart transplantation model). Large animal models are of primary interest for the surgeons as they allow the use of original equipment developed for human patients, imitating procedures used in cardiovascular surgery. Advanced as they are, these models possess a number of limitations as well. Larger animal models are expensive and complex. Positive results obtained using young and healthy animals to mimic the clinical situation often cannot be reproduced in clinics where the average age of patients is above 65-70 years. The negative outcome of the recent blinded randomized multicenter clinical trials testing cardioprotective efficacy of drugs which showed an impressive cardioprotective potential in large animal models was one of the recent disappointments revealing the limitations of large animal studies (Bolli *et al.*, 2004). Therefore, more attention was recently paid to the improvement of isolated organ models enabling usage of the organs of aged and diseased rodents without causing unnecessary distress to the animals.

4.6 Oxygenator and technical characteristics of the perfusion circuit

A new miniaturized hollow fibre oxygenator (Fig 2) designed in our lab by Prof. J. Vogel in the Institute of Veterinary Physiology is a key element of the circulation circuit used for the perfusion of the rat heart with autologous blood. The total filling volume of this system is reduced from ≥100 ml, as common for the conventional Langendorff settings, to 5 ml. The same system was further miniaturized and adapted to a perfusion volume of ~0.5 ml for the mouse heart perfusion setup. Funded by the 3R Research Foundation, my thesis focused on validation of the new perfusion setup and its capacity to **R**eplace the animals used to assess basal cardiovascular toxicity of drugs as well as in pilot studies on responses of the heart to

severe hypoxia or ischemia/reperfusion, **Reduce** the number of tests performed *in vivo*, and **Refine** experimental conditions of these final tests.

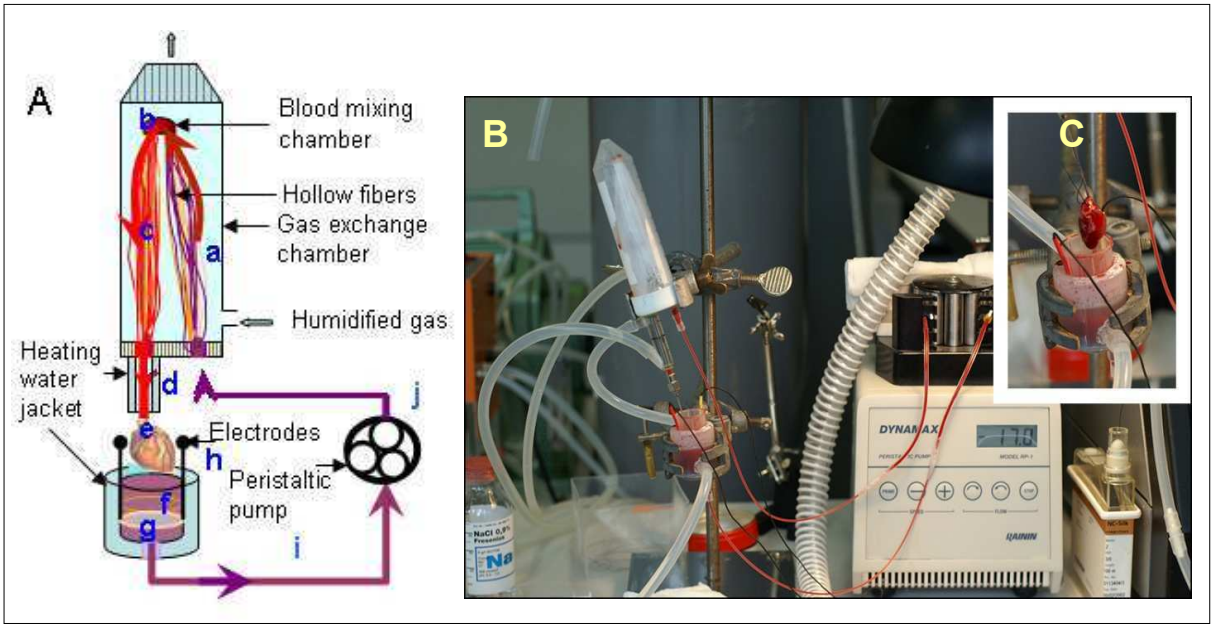

Figure 2: Schematic representation of the hollow fibre oxygenator and the perfusion circuit.

Legend:

A: Perfusion circuit scheme including the following components: (a) gas exchange chamber, (b) blood mixing chamber; (c): fibres permeable for gas and impermeable to water; (d) thermostat; (e) cannula with the heart mounted; (f) organ chamber equipped with an anti-clot filter (g) and ECG/heart rate electrodes (h); (i) PV tubing with holders; (j) peristaltic pump

B: Frontal view on the oxygenator and the organ chamber

C: Perfusion circuit with the peristaltic pump and the organ chamber with the mounted heart

4.6.1 Design of the hollow fibre mini oxygenator

Mammalian lungs have a large interface area and high permeability to gases, allowing efficient gas exchange. The miniaturized oxygenator (Fig 2) was designed by Prof. Vogel as a part of a new rodent “heart-lung machine” prototype mediating O_2/CO_2 exchange with minimal damage to the circulating blood cells. Care was taken to avoid foaming or clot formation (Wegner, 1997). Structure and thickness of the fibres were selected to enable sufficient gas exchange within seconds (Iwahashi *et al.*, 2004).

The material which was used in construction of the mini oxygenators (Oxyphan® PP 50/200 Membrane GmbH, maximal pore size ≤0.2 µm, wall thickness 50 µm, diameter 200 µm, length reduced to 12 cm) has been designed for the human heart-

lung machines (Fig 3). These fibres are permeable to gases but impermeable to liquids.

In contrast to that for *in vivo*, in many oxygenators, blood was passing through the lumen of the fibre, whereas humidified gas was pumped through the gas exchange reservoir in contact with the external surface of the fibres (Fig 3). “Venous” blood passes though the in-coming bundle consisting of ~150 fibres for the rat setup (10 fibres for the mouse setup), as shown in Fig. 2 (a), into the mixing chamber (b). Thereafter partially reoxygenated blood is forwarded through the exiting fibre bundle (c) into the heating chamber (d) and further into the cannula on which the heart is mounted (e). The perfusion chamber is equipped with circular electrodes to enable heart rate and ECG recordings during the perfusion. Temperature of the water jackets is adjusted so that the temperature in the exiting blood is maintained at 37°C. After passing through the coronary vessels, blood is collected in the water-jacketed organ chamber (f) and from there, pumped with a peristaltic pump (i) back into the oxygenator. Clots, if formed, are stopped from re-entering the perfusion circuit by a filter mesh (pore size 200 μm^2 for the rat system and 10 μm^2 for the mouse setup).

The ECG comparable to aVL or II projections and the beat-to-beat calculated heart rate were recorded with a Heart Rate Modul (Hugo Sachs Elektronik) processed by a PowerLab analogue-digital transducer (AD Instruments) and stored as digital files for offline analysis.

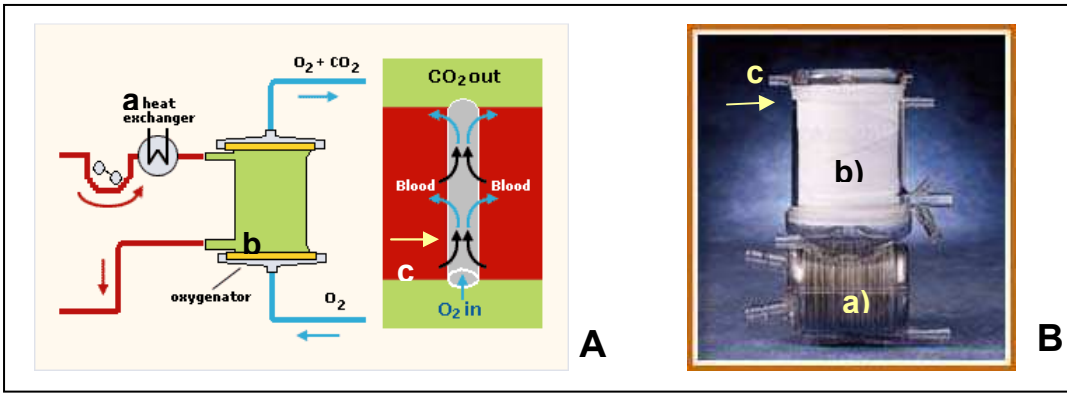

Figure 3: Human “artificial lung”.

Legend:

A: Oxygenator scheme from www.meditronic.com. AFFINITY® NT

B: The original design

a: Heat exchanger

b: Gas exchange interface

c: Perfusion fluid

The left ventricular pressure in rat hearts was measured with hand-made catheter balloons (Fig 8) made of PVC film used for fresh food wrap and syringe needles attached via a polyethylene catheter (outer diameter: 0.96 mm) to a piezoelectric pressure transducer. The balloon was introduced into the left ventricular cavity via left atrium incision section. Then the balloon was gently inflated (~500 µl filling volume) to gain a pre-load of 1-5 mmHg. The pressure was continuously recorded using the PowerLab.

The gas mixture used to saturate the circulating blood with oxygen contained 5% CO_2, 20% O_2 and 75% N_2. The temperature was adjusted to 37°C and it was passed through the humidifier. The peristaltic pump (Dynamax, Ranin), in most cases, was adjusted to maintain a constant blood flow of 3 ml/min through the perfusion circuit mimicking *in vivo* rat heart coronary blood perfusion conditions. For the mouse perfusion setup, perfusion was adjusted to 0.8 ml/min.

The blood volume necessary for one experiment was 5-8 ml for the rat and at least 0.5 ml for the mouse perfusion circuit. In order to keep the perfusion system air-free and avoid thromboembolic events, the system was pre-filled with perfusion buffer of the following composition: 120 mM, NaCl: 25 mM, $NaHCO_3$: 1 mM, $CaCl_2$: 0.15 mM, $MgCl_2$: 10 mM, glucose: 10 mM, TRIS-HCl (pH 7.4). Before heparinisation, blood was introduced in the system. As a result, the haematocrit of the final blood-buffer mixture ranged between 25% and 30%.

4.6.2 Animal handling and tissue harvesting

Rat: Male Wistar rats weighing 180-250 g (Elivage Javier) were used for the experiments. Animal keeping and experimentation were performed in accordance with Swiss animal protection laws and institutional guidelines. The rats were anesthetized using isofluorane (3% in a 1:1 mixture of O_2 and N_2O) and the abdominal cavity was opened. Heparin (~100 µl of 10'000 U/ml heparin, Brawn AG) was injected into the vena cava and thereafter 5-8 ml blood was collected. Rats were decapitated and the heart harvested was immediately placed in ice cold heparin-containing perfusion buffer.

Mouse: Approximately two-month-old C57BL6 mice (local breeding) were anesthetized using isofluorane, and the abdominal cavity was opened. Heparin (~100

µl) was injected into vena cava and thereafter, 1-2 ml blood was collected. Due to the increased risk of blood coagulation, mice received 100 µl of heparin subcutaneously 30 minutes prior the experiment. The heart was harvested and immediately placed in the ice cold heparin-containing perfusion buffer.

4.6.3 Heart mounting procedure and onset of perfusion

Rat: After harvesting the heart, the aorta was isolated and the heart was mounted onto the cannula with the aorta sealed with suture (Fig 2). Care was taken to avoid thromboembolism and not to damage the aortic valve and the aortic walls. The time between harvesting and the onset of the perfusion never exceeded 5 minutes. At the time of mounting, the perfusion circuit was filled with oxygenated blood at room temperature. Perfusion was initiated at a rate of 3 ml/min, and the blood was gradually warmed to 37°C.

Mouse: Excision and aortal isolation were performed under the binocular microscope. Mouse heart mounting required two pairs of hands. Perfusion with blood at room temperature was initiated at a flow rate of 0.8 ml/min and the temperature was gradually increased to 37°C.

4.7 Validation of the model

4.7.1 Blood properties in the perfusion circuit

Blood gas content before and after introduction into the perfusion circuit was controlled using a blood gas analyser (phOx Nova plus, Nova Biomedical); the haematocrit was additionally assessed using microcapillaries, and haemolysis was measured with a Nanodrop 200 spectrophotometer (Thermo Scientific, Witec AG). Glucose levels in plasma were measured using a glucose meter and enzyme strips (Ascensia Elite Bayer).

4.7.2 Water evaporation

Rat: As mentioned above, blood haematocrit at the onset of the heart perfusion was somewhat lower than that *in vivo*: 30.69±11.72% vs. 24.25±16.22% in rats (mean

±SD, N=11). This dilution did not compromise O_2 delivery to the myocardial tissue, but reduced blood trauma and clotting risks. Haematocrit values gradually increased with time due to gradual water evaporation from the organ chamber. This dehydration could be compensated by extra water supplementation aliquots at 40 µl/20 minutes.

4.7.3 Plasma glucose depletion

Rat: The glucose consumption by erythrocytes and myocardium resulted in a gradual deprivation of plasma glucose pools during perfusion. The glucose rate was assessed for the rat perfusion system (Fig 4). Based on the glucose consumption assessment, we have developed a protocol which avoids glucose deprivation along with water loss compensation. Glucose utilization by rat erythrocytes is found to be much higher than in hearts, 0.119±0.003 µmol/(gHb*min) (Hashimoto *et al.*, 1996) and 0.03-0.05 µmol/(g*min) (Kuschinsky *et al.*, 1993), respectively. Therefore, continuous glucose supply was necessary over time. Glucose consumption in rat blood in our system (0.60 µmol/gHb/min) was about five times higher than what has been reported in the literature. Glucose deprivation and loss of water from the perfusion circuit could be compensated by adding 40 µl aliquots of 140 mM glucose solution in water to the system every 20 minutes. The glucose and physiological solution were filtered through a 5 µm filter prior to use.

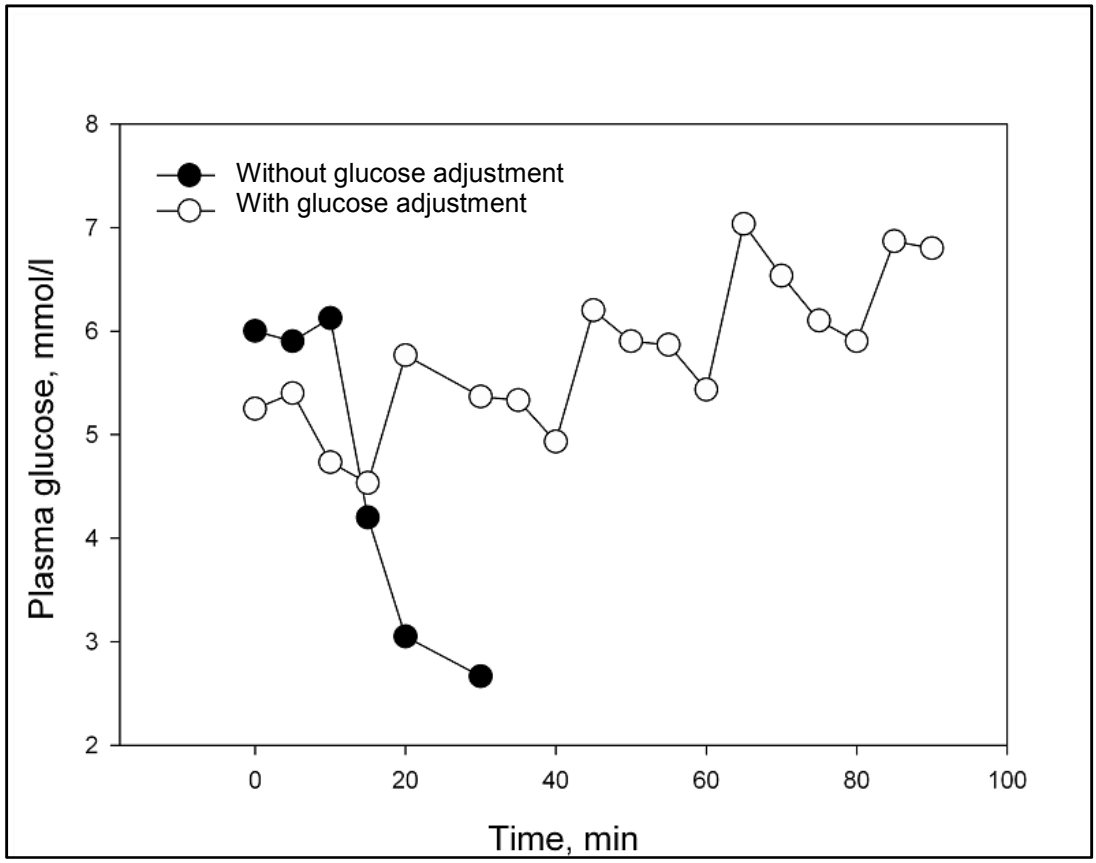

Figure 4: Rat plasma glucose levels with and without glucose adjustment. Aliquots (40 µl of 140 mM glucose stock solution) were added to the circulating blood each 20 minutes.

Mouse: Plasma glucose levels were continuously monitored during perfusion and 2 µl of glucose solution (140 mM) was added whenever glucose levels declined. Glucose utilisation in this system was significantly lower compared to that in rat blood and in heart blood-perfused heart system.

4.7.4 Haemolysis

Special attention was given to the monitoring of blood trauma during the passage of erythrocytes through the perfusion system. Haemolysis was monitored in both rat and mouse blood over the course of perfusion (Fig 5) as haemoglobin and K^+ release into plasma (Fig 5: A). Both factors may interfere with the heart function. Haemoglobin is a potent NO scavenger (Ascenzi & Visca, 2008) causing vasoconstriction, and free iron when liberated from haeme may cause oxidative stress in the endothelium by catalysing the Fenton reaction. An increase of plasma K^+ results in depolarisation and may cause stunning when exceeding 7-8 mM.

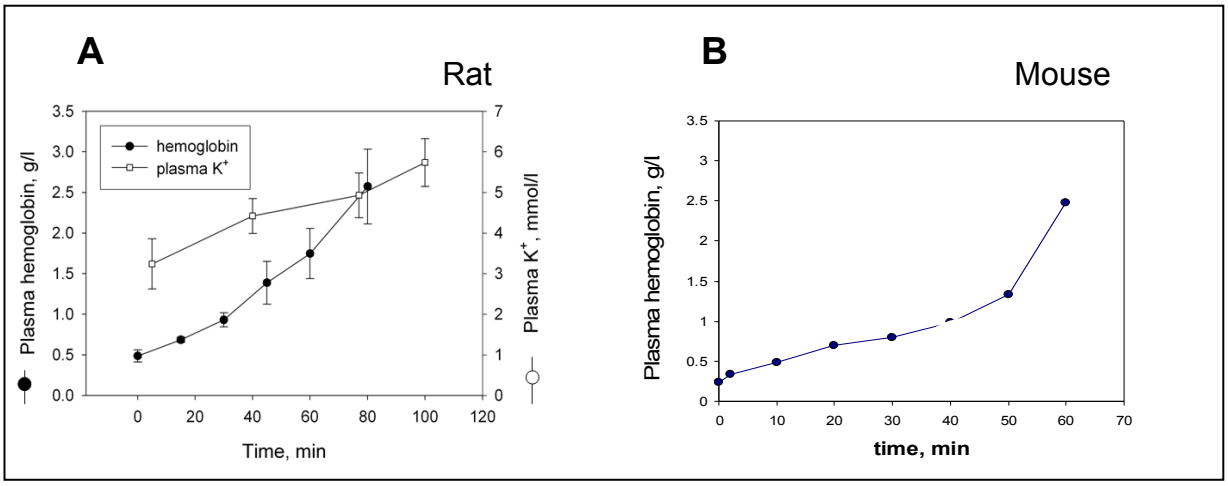

Figure 5: Blood trauma assessed in the rat and mouse perfusion circuits.

Legend:

A: Haemolysis in rat perfusion setup assessed as an increase in plasma K^+ and extracellular haemoglobin accumulation.

B: Haemolysis accessed in mice blood as plasma haemoglobin accumulation.

Similar problems are well-described in clinical settings when human blood is processed by heart-lung machines. High shear stress, turbulence, and dilution of plasma causes haemolysis (Kameneva *et al.*, 1999) (Watanabe *et al.*, 2007) (Leverett *et al.*, 1972; Paul *et al.*, 2003). Moreover, contact of cells with non-biological surfaces facilitates thrombosis and cellular damage (Sutera, 1977). Among preventive measures undertaken to reduce haemolysis are the careful selection of the materials used in construction of the oxygenator and perfusion circuit as well as special construction of the peristaltic pumps to reduce blood trauma.

Both free plasma haemoglobin and excessive K^+ are removed from human blood passing through the heart-lung machine by introducing a filter. Plasma K^+ is then supplemented to adjust the concentration to 3-4 mM. The extremely low volumes involved in our systems make this manipulation technically impossible. Thus, perfusion time in the rodent settings should be limited to 60-80 min. When necessary, longer perfusion times may require gradual replacement of blood used for perfusion. One adult rat may provide up to 15 ml blood from which 5-6 ml are required to prime the perfusion and fill the circuit. The rest may be stored for future use.

4.7.5 Efficiency of blood oxygenation

The oxygenator blood-gas interface areas (540 cm^2 for the rat oxygenator and 36 cm^2 for the mouse oxygenator) were significantly smaller than those of the lung capillary surface area (~5000 cm^2 for a rat and ~590 cm^2 for a mouse) (Soares S, 2002). The following experiments were performed to assess the gas exchange efficiency of the mini oxygenator.

Based on the findings obtained (Fig 6 A), two minutes were assessed to be sufficient to reach steady-state levels of haemoglobin O_2 saturation. In the next experimental set, rat blood was allowed to pre-equilibrate with a humidified gas phase containing 5% CO_2, 25% O_2 and 70% N_2. Thereafter, O_2 levels in the gas phase were decreased in intervals (20%-15%-10%-5%-3%-1% down to a nominally O_2-free gas mixture). The gaseous phase always contained 5% CO_2. The perfusion rate was fixed at 3 ml/min. Blood samples were collected at the end of the second minute of perfusion at a fixed pO_2 in the gaseous phase, and OxyHb levels were assessed. The resulting rat haemoglobin O_2 saturation curve (SO50% of ~7% O_2) is shown in Fig 6 B. The SO50% obtained matches the values (~10% O_2) reported earlier (Julien *et al.*, 2006). The data obtained suggest that despite reduced blood gas interface area, the hollow fibre oxygenator efficiently performs gas exchange within a relatively short period of time.

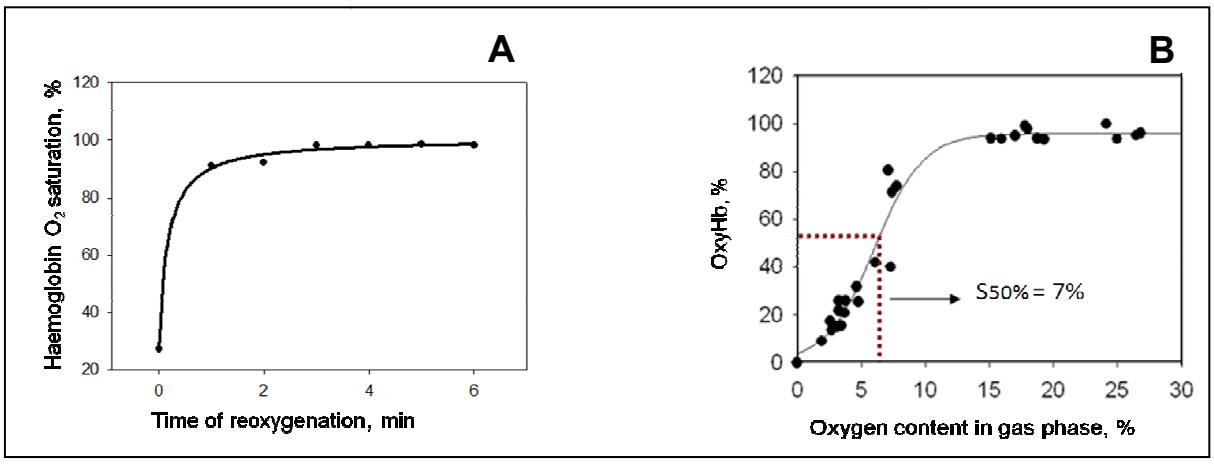

Figure 6: Blood oxygenation in the hollow fibre oxygenator.

Legend:

A: Reoxygenation kinetics at 3 ml/min perfusion rate

B: Haemoglobin oxygen saturation of rat blood as a function of pO_2 in gas phase at 3 ml/min

4.8 Heart function during long-term perfusion

4.8.1 Heart rate

The spontaneous heart rate assessed in an isolated blood perfused organ remained stable over an hour. As the temperature of the organ gradually increased from ~ 4°C to 37°C, the heart rate increased and stabilized at 37°C (Fig 7A). The spontaneous heart rate ranged between 200 and 380 beats/min which was lower than that reported in the conscious animal (400-480 beats/min) and similar to that in Langendorff preparations (250-320 beats/min) (Sutherland & Hearse, 2000) or in anesthetized animals (320-380 beats/min) (Hayashi, 2003). In some senescent animal hearts, arrhythmias and stunning were observed. However, deoxygenation often (up to 80%) resulted in development of arrhythmia. An example of stunning caused by hypoxic treatment in the senescent heart is shown in Fig 7 B.

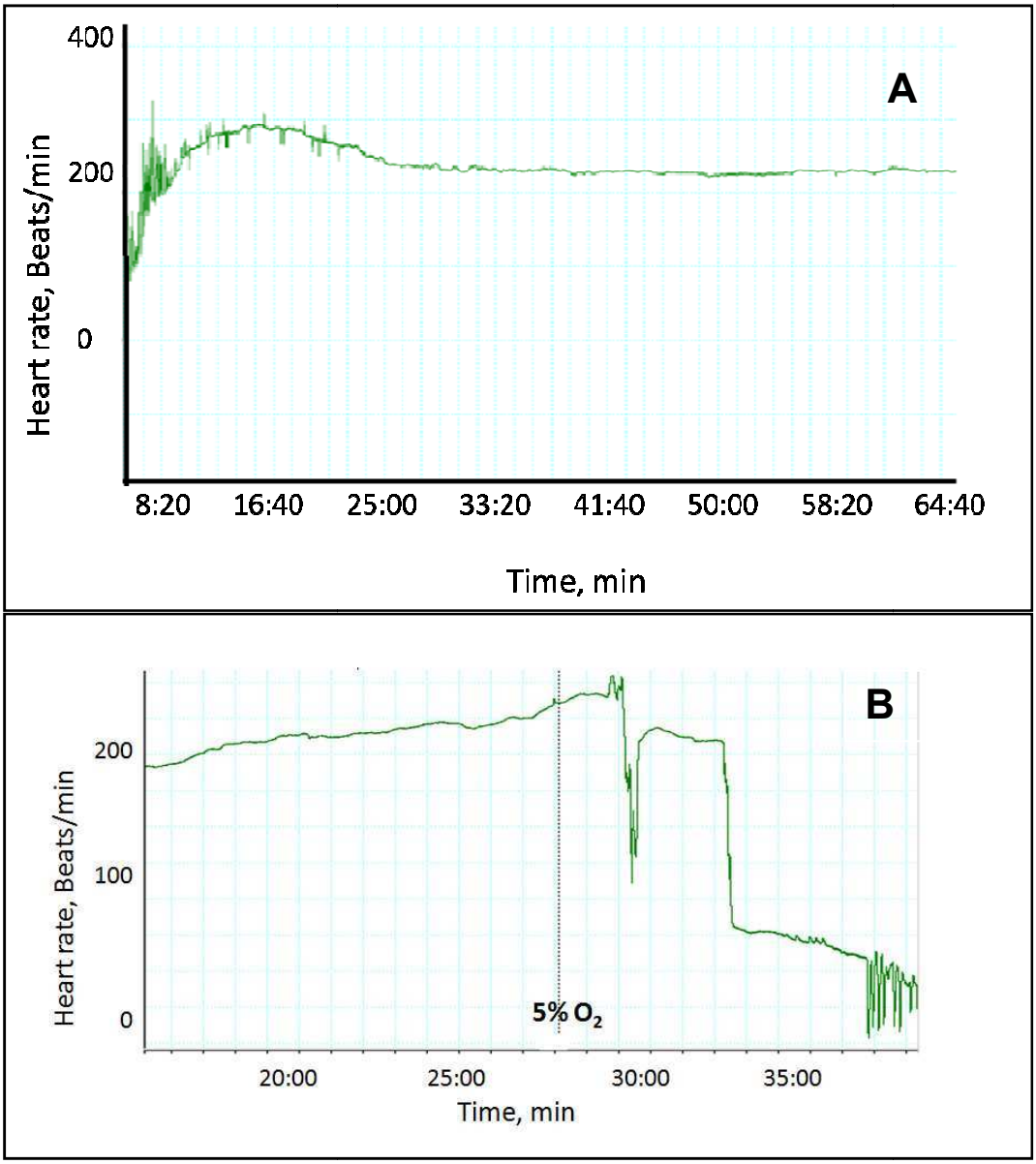

Figure 7: Heart rate original recordings in spontaneously beating heart.

Legend:

A: No arrhythmias were observed during 3-4 hours

B: Stunning in senescent rat heart in response to reduction in haemoglobin oxygen saturation from 98% to 35%

4.8.2 Left ventricular pressure

The left ventricular pressure (LVP) was assessed in rat hearts with a self-manufactured balloon catheter using a syringe needle and a 25 mm diameter piece of kitchen wrap according to the instructions provided by ADInstruments

(www.adinstruments.com). The balloon was attached to the pressure transducer, and the bridge amplifier was filled with 0.9% NaCl and introduced into the left ventricular cavity via the left atrium. After adjusting basal LVP (~8 mmHg-systole) by filling the balloon, this parameter was monitored continuously.

The original recording shown in Fig 8 was taken during the 2 h 40 min perfusion of the heart isolated from a 1.5-month-old male Wistar rat. As can be seen from the recording, the average LVP values observed (80-90 mm Hg at systole) were within the physiological range (Neely *et al.*, 1967).

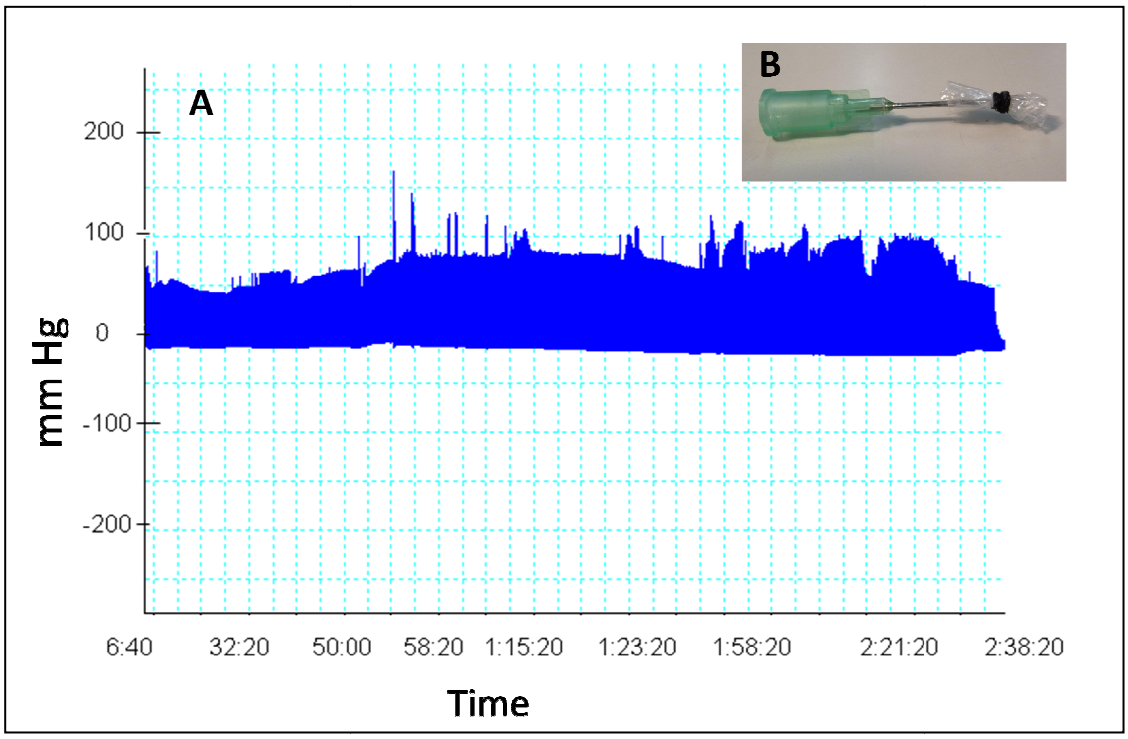

Figure 8: Left ventricular pressure in the perfused rat heart.

Legend:

A: Recorded pressure developed by the blood-perfused organ remained stable over two hours

B: Image of the self constructed balloon catheter

4.8.3 ECG measurements

During perfusion, ECG (aVL projection) was continuously recorded with a Heart Rate Modul (Hugo Sachs Elektronik) attached to the PowerLab analogue-digital transducer (PowerLab AD Instruments). The data were stored as numeric files and analysed manually. Figure 9 represents the ECG from the rat heart (panel A) which resembles that monitored *in vivo* (panel B). Analysis revealed that the action potential

duration as well as the QT intervals in the isolated heart were within the range reported *in vivo* (Table 3).

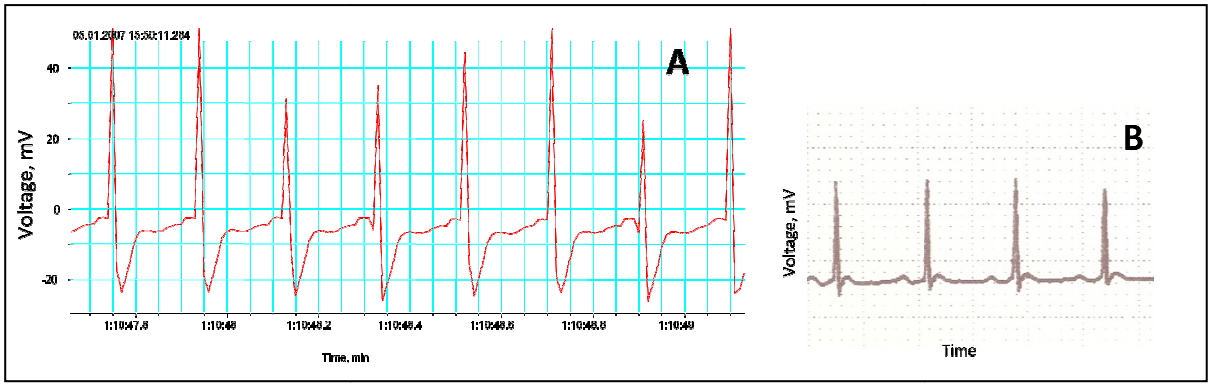

Figure 9: ECG measurements.

Legend:
A: ECG recording after 1 h 10 min of perfusion of the isolated rat heart with autologous blood
B: *In vivo* original record of an ECG aVL projection in a rat

Table 3: Rat ECG parameters *ex vivo* and *in vivo*

Parameter	**Blood-perfused heart**	***In vivo* values**
QRS, msec	21-43	40-55
PQ, msec	27-50	60
AD, msec	130-150	150-200

4.9 Heart biochemistry

4.9.1 Methodology

After 1 h of perfusion, rat hearts were cooled down and washed free of blood with ice-cold isotonic sucrose buffer and frozen in liquid nitrogen for the detection of Na, K-ATPase activity, ATP and GSH in tissue. Samples for the tissue ion and water content were collected into pre-weighed, pre-dried tubes and processed as mentioned below.

The tissue water and ion content were monitored in blood-free ventricular samples. The difference in weight of the tissue samples before and after drying at 80°C as compared to the constant weight was considered to be the tissue water and was expressed as a percentage of the wet weight of a sample. After drying, the samples

were wet-burned with ultra-pure concentrated HNO_3. Tissue Na^+ and K^+ content were then determined using flame photometry (IL- 943, Instrumentation Laboratory, Bedford, MA, USA). The results obtained were normalized to the dry weight of the sample.

The tissue GSH, GSSG and ATP levels were assessed in blood-free ventricular tissue preparations. Frozen ventricular fragments (~0.1 g) were homogenized on ice in KCl-MOPS buffer (100 mM KCl and 10 mM MOPS, pH 7.4 and deproteinized with 5% trichloroacetic acid). After centrifugation (5 min, 9000·g, at 4°C), an aliquot of the protein-free supernatant was neutralized to pH~7 with TRIS-OH powder, and ATP was assessed using an ATP Bioluminescent Assay Kit (FLAA, Sigma, St Louis, MO, USA). The luminescence intensity in the heart tissue samples and in standard the samples of known ATP content was monitored using a Sirius luminometer (Berthold Detection Systems). Values were normalized to tissue wet weight. GSH and GSSG were assessed in the protein-free supernatant using Ellmann's reagent and also normalized to sample wet weight.

Hydrolytic activity of the Na,K-ATPase was determined in ventricular tissue homogenates from the isolated blood-perfused hearts exposed to normoxia or hypoxia (20 or 5% O_2 in gas phase) for 60 minutes. Na,K-ATPase hydrolytic activity was assayed as previously described (Rathbun & Betlach, 1969). In brief, ventricular tissue was homogenized in KCl-MOPS buffer and an aliquot was added to the medium containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 1 mM ouabain when mentioned (37°C, 10 min). In the presence of these saturating concentrations of the Na,K-ATPase substrate and ligands, the measured enzymatic activity corresponds the pseudo-maximal ATP cleavage rate, pseudo V_{max}. Na,K-ATPase hydrolytic function was assessed as the difference in ATP cleavage rate in the presence and absence of 1 mM of blocker, ouabain. The enzymatic ATP hydrolysis was initiated by adding ATP-HEPES-NaOH mixture (final concentrations in the medium were 3 mM and 30 mM, respectively) and allowed to proceed for 7 minutes, after which it was stopped by adding the ice-cold stopping solution (4% formaldehyde in 1.3 M sodium acetate buffered with acetic acid to pH 4.3). The samples were mixed with 100 µl of $SnCl_2$ solution (15 mg $SnCl_2$ in 5 ml of 0.002% acetic acid) and 100 µl of 2% $(NH_4)_2MoO_4$ solution in distilled water. After 15 min, a coloured complex of phosphate with Sn^{2+} and $(MoO_4)^{2-}$ was formed and was evaluated by measuring the optical

density (660 nm, Lambda 25 spectrophotometer, Perkin Elmer). Blank samples contained no cell lysates or the lysates were added after the ATP hydrolysis was stopped. The hydrolytic activity of Na/K ATPase was calculated as a difference in the rate of phosphate production in the corresponding ouabain-free and ouabain-containing sample pairs. Activities were normalized to the amount of protein in the homogenates, and quantified using the Bio-Rad protein assay (Bio-Rad Laboratories Inc., USA).

4.9.2 Metabolism and tissue ATP levels

The ATP demands of the isolated unloaded heart were lower than those observed *in vivo*. As a result, ATP levels measured in the myocardium *ex vivo* exceeded those obtained in freshly isolated myocardium (5.2±1.1 vs. 3.01±0.63 µmol/g wet weight respectively, $p<0.05$). The values obtained for the glucose utilization rate in the isolated heart (20.23±4.47 µmol/(100g*min)) measured by means of ^{14}C-DOG autoradiography were slightly lower than those *in vivo* (30-50 µmol/(100g*min)) obtained with the same technique (Kuschinsky *et al.*, 1993).

4.9.3 Water and ions

Ta data presented in Table 4 reveal the changes in biochemical parameters of the myocardial tissue that could follow 90 minutes of rat heart perfusion with well-oxygenated autologous blood compared to those in a fresh-isolated organ.

Biochemical parameters listed in Table 4 were assessed in isolated blood-perfused rats. Among those parameters are reduced (GSH) and oxidized (GSSG) glutathione, and ATP levels. The Na^+ and K^+ content were measured in ventricular tissue as described.

The ex vivo perfusion did not affect the ion and water content of ventricular tissue. However, it resulted in the development of oxidative stress which is due to a reduction in GSH and accumulation of GSSG.

Table 4: Water and ion measurements in rat hearts

Parameter	*ex vivo*	*in vivo*
Water (%)	72.51 ±0.72	74.1 ±0.2
Na^+(mmol/Kg dw)	43.28 ±16.29	49.2 ±2.6
K^+(mmol/Kg dw)	269.44 ±21.32	285.0 ±7.1
GSH (µmol/g ww)	1.51 ±0.36	2.223 ±0.059
GSSG (µmol/g ww)	0.104 ±0.039	0.44 ±0.21*

* $p < 0.05$

4.9.4 Redox state

Excessive amounts of oxygen in blood saturated with 21% O_2-containing gas mixture caused tendency to moderate oxidative stress in the myocardium.

4.10 Our model of choice

The autologous blood-perfused rodent heart model is a promising technique in the field of cardiovascular research and it was of crucial importance to our study. It closely mimics the *in vivo* conditions without promoting animal suffering, and therefore adheres to the 3R's principle. This model fills the niche between the cellular and *in vivo* levels of complexity (Table 1).

The validation of the model demonstrated that the function of the isolated blood-perfused heart (HR, LVP and ECG as well as the ion/water balance) and some of the basic biochemical parameters in myocardial tissue remained stable for at least 90 minutes of perfusion and did not deviate significantly from the *in vivo* values. The oxygenation of the organ is sufficient to support its autonomous function. This model may be further used to assess the effects of drugs and treatments under controlled standardized conditions (perfusion rate, drug concentrations, temperature regiment).

Particularly in this study, the use of this method allowed us to further increase hypoxic stress in rat hearts under conditions that wouldn't be possible in *in vivo* experiments. Furthermore, this was a precious tool, which permitted us to better understand acute cardiac stress in the absence of sympathetic and cholinergic effects.

5. Model application: Myocardial responses to hypoxia

5.0 Motivation and goals

We used the isolated blood perfused heart model to monitor autonomous heart responses to hypoxia. Metabolic enzymes (Lopaschuk *et al.*, 2010), cation channels (Hool, 2005), redox state (Park *et al.*, 2007) and calcium handling (Pravdic *et al.*, 2009) in the heart are known to respond rapidly to the changes in the myocardial oxygen supply. Oxygen consumption in the heart exceeds that of the brain (Dobson, 2004). Having a mass of about 0.4% of the total mass of the human body, the heart is responsible for around 16% of total oxygen consumption. Reduction in oxygen supply leads to a reduction in cardiac output (Calbet *et al.*, 2009) and arrhythmias (Keating & Sanguinetti, 2001) which in turn are the cause of a high number of deaths and hospitalisations (Madsen *et al.*, 2007).

5.1. Objectives

Objectives of the study performed using the new experimental system were:

1. Characterization of the isolated rat heart responses to acute global hypoxia and comparison of the observed findings with those obtained for the systemic *in vivo* hypoxic model.
2. Assessment of the possible interventricular variation in hypoxic responses.

Among the parameters monitored were functional parameters (heart rate and ECG), biochemical parameters (glucose utilization rate (Kuschinsky *et al.*, 1993), tissue ATP (Petrushanko *et al.*, 2006), Na^+/K^+ and water content (Tanonaka *et al.*, 1999) and the hydrolytic activity of Na,K ATPase (Rathbun & Betlach, 1969; Bogdanova *et al.*, 2005), as well as the tissue redox state (reduced to oxidized glutathione ratio and half-cell redox potential Ehc for this redox couple. ($Ehc = -240 - (59.55/2)\log(GSH)^2/[GSSG]$)) (Tietze, 1969) (Schafer & Buettner, 2001). Tissue biochemical parameters were assessed in the right (RV) and the left (LV) ventricle separately.

In vivo hypoxic exposure was performed in the InVIVO 1000® hypoxic cabinet. The animals (male Wistar rats 180-250 g) were exposed to 10% oxygen for 1 hour,

sacrificed and the heart collected. *Ex vivo* hypoxia was performed using the heart and blood of sex and age-matched animals. Isolated hearts were perfused with autologous blood equilibrated with a gas phase containing either 21% (normoxia) or 5% (hypoxia) O_2 at 37°C for 1 hour. Heart rate and ECG (aVL projection) in isolated perfused hearts were recorded with a Heart Rate Modul (Hugo Sachs Elektronik) attached to the PowerLab analogue digital transducer (PowerLab AD Instruments). Data were stored as numeric files and analyzed manually using Microsoft-Excel software.

The data obtained were summarized in a paper recently published in the American Journal of Physiology (see following chapter).

6. Manuscripts

6.1. Manuscript

Interventricular heterogeneity in rat heart responses to hypoxia: the tuning of glucose metabolism, ion gradients, and function

Published at: American Journal of Physiology, 2011

Interventricular heterogeneity in rat heart responses to hypoxia: the tuning of glucose metabolism, ion gradients, and function

Milena Segato Komniski, Sergej Yakushev, Nikolai Bogdanov, Max Gassmann and Anna Bogdanova

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Am J Physiol Heart Circ Physiol 300: H1645–H1652, 2011.
First published March 11, 2011; doi:10.1152/ajpheart.00220.2010.

Interventricular heterogeneity in rat heart responses to hypoxia: the tuning of glucose metabolism, ion gradients, and function

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Segato Komniski M, Yakushev S, Bogdanov N, Gassmann M, Bogdanova A. Interventricular heterogeneity in rat heart responses to hypoxia: the tuning of glucose metabolism, ion gradients, and function. *Am J Physiol Heart Circ Physiol* 300: H1645–H1652, 2011. First published March 11, 2011; doi:10.1152/ajpheart.00220.2010.—The matching of energy supply and demand under hypoxic conditions is critical for sustaining myocardial function. Numerous reports indicate that basal energy requirements and ion handling may differ between the ventricles. We hypothesized that ventricular response to hypoxia shows interventricular differences caused by the heterogeneity in glucose metabolism and expression and activity of ion transporters. Thus we assessed glucose utilization rate, ATP, sodium and potassium concentrations, Na, K-ATPase activity, and tissue reduced:oxidized glutathione (GSH/GSSG) content in the right and left ventricles before and after the exposure of either the whole animals or isolated blood-perfused hearts to hypoxia. The hypoxia-induced boost in glucose utilization was more pronounced in the left ventricle compared with the right one. ATP levels in the right ventricle of hypoxic heart were lower than those in the left ventricle. Left ventricular sodium content was higher, and hydrolytic Na, K-ATPase activity was reduced compared with the right ventricle. Administration of the Na, K-ATPase blocker ouabain caused rapid increase in the right ventricular Na^+ and elimination of the interventricular Na^+ gradients. Exposure of the hearts to hypoxia made the interventricular heterogeneity in the Na^+ distribution even more pronounced. Furthermore, systemic hypoxia caused oxidative stress that was more pronounced in the right ventricle as revealed by GSH/GSSG ratios. On the basis of these findings, we suggest that the right ventricle is more prone to hypoxic damage, as it is less efficient in recruiting glucose as an alternative fuel and is particularly dependent on the efficient Na, K-ATPase function.

glucose utilization; redox state; ventricles

BOTH THE LEFT VENTRICLE (LV) and the right ventricle (RV) possess structural, biochemical, and metabolic differences that meet their functional requirements. The LV has approximately three times the mass and twice the wall thickness of the RV and can be viewed as a "pressure pump" whose cavity resembles an elongated cone. The RV, which pumps at a lower pressure and operates as a "volume pump" is crescent-shaped (26). The double-peaked waveform of both the right ventricular pressure and the right ventricular outflow recorded using the electrically isolated right ventricular free-wall preparation, revealed the presence of two components in the RV contractile function (26). The first is attributed to the contraction of the free wall of the RV, whereas the second is related to the LV and its septal contraction. Analysis of numerical data suggests that ~30% of the stroke output of the RV was generated by the LV (15).

These differences in the generation of force are mirrored by differences in the properties of the force-generating proteins. The distribution of myosin heavy-chain (MHC) isoforms between the ventricles supports this functional asymmetry. The RV of the rodent heart is enriched with a fast α-MHC isoform that exhibits higher levels of myosin ATPase activity than the slow β-MHC isoform (8, 31, 42). The slow muscle fiber-specific energy-saving β-MHC isoform, which requires less energy to generate cross-bridge force, is correspondingly more abundant in the LV than in the RV (21, 34).

Heterogeneity in ventricular force-generation capacity is further supported by the differences in sarcoplasmic reticulum Ca^{2+} reserve (27) between the two ventricles. Excitation propagation differs between the ventricles as do Ca^{2+}-dependent outward transient $I_{to,f}$ currents(36) and ATP-dependent, non-voltage-gated rectified K^+ currents (2, 19). These differences stem from heterogeneity in channel expression levels [e.g., that of Kv4.2, Kv4.3, KChIP2, Kv1.5, and Kv2.1 (9, 25)], densities, amplitudes, and their sensitivity to agonists (1, 25, 27, 35, 41). Loss of heterogeneity in ion-current densities after myocardial infarction results in the development of arrhythmias (25). Interventricular differences in channel-mediated passive K^+ transport imply that active transport of K^+ mediated by the Na, K-ATPase also varies between the ventricles to sustain transmembrane ion gradients. However, local differences in the abundance and activity of Na, K-ATPase in ventricular tissue have never been studied.

Heterogeneity in energy demand required for sustaining the contractile force and ion gradient preservation suggests asymmetry in energy production in the ventricles. A local difference in oxygen extraction (60–75% by the LV and 50–51% by the RV) was observed during coronary venous blood sampling in open-chest dogs (28, 49). The right and left resting coronary venous Po_2 is ~30 and ~20 mmHg (4.0 and 2.7 kPa), respectively, indicating higher demand for energy in the LV (49). Under resting conditions, oxygen supply is controlled by coronary blood flow, which is higher in the left coronary artery than in the right.

What happens when the oxygen supply becomes limited? Severe local or global hypoxia results in "myocardial hibernation" followed by reduction in heart rate and myocardial contractility (17). Suppression of oxidative phosphorylation fuelled mainly by fatty acid metabolism is at least partially compensated for by an increase in anaerobic glycolysis to avoid irreversible ATP deprivation (11, 12, 20, 44). In a single study, autoradiography was used to assess local glucose utilization rates in the ventricles of conscious rats (29). Data

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obtained in these in vivo settings indicate the existence of LV-to-RV heterogeneity in glucose utilization, which cannot be statistically resolved because of high interindividual variation. The present study explores the mechanisms of interventricular heterogeneity in response to acute hypoxic challenge in rat hearts. Acute challenge (1 h) was employed to exclude the effects driven by the changes in gene expression. We hypothesized that the differences in oxygen supply and anaerobic metabolism, as well as those in expression and activity of ion transport systems, will result in differential sensitivity of ventricles to oxygen deprivation.

In vivo and ex vivo experimental models were used to compare systemic and autonomous responses of the ventricular tissue to reduced oxygen supply, respectively. Data obtained in animals exposed to hypoxia for 1 h were compared with those generated in isolated rat hearts perfused with hypoxic autonomous blood passing through a hollow fiber oxygenator. By the reduction of the complexity of the system, this ex vivo model offered greater precision and reproducibility. We have monitored the interventricular heterogeneity and hypoxia- sensitivity of glucose utilization, tissue ion content, and redox state. Resulting data revealed substantial differences in glucose utilization capacity and maintenance of Na^+/K^+ gradient between the LV and RV.

MATERIALS AND METHODS

Organ harvesting procedure. Male Wistar rats (180–250 g) were purchased from Janvier (Le Genest, St Isle, France). All animal experiments were approved by the Federal Veterinary Office and performed in accordance with Swiss animal protection laws and institutional guidelines that comply with guidelines of the American Physiological Society and the Institute of Laboratory Animal Resources.

In vivo hypoxic model. Rats exposed to systemic hypoxia were placed in an $InVIVO_2$ 1000 hypoxic cabinet (Ruskinn Technology/Ruskinn Life Sciences, Bridgend, UK) in standard cages. Food and water were provided ad libitum. Ten percent oxygen was used in the in vivo hypoxic settings because conscious rats can tolerate this O_2 levels well and show a prominent hypoxic response (46, 47). During the experiments, rats showed no signs of distress except for reduced activity and moderate hyperventilation. The reduction of O_2 content in the hypoxic chamber from 20% to 10% was performed gradually, reducing oxygen in 2% increments with adaptation periods of 10 min at each pO_2 level. The animals remained in the hypoxic chamber for 1 h and were euthanized immediately upon removal from the chamber. The hearts were quickly harvested and chilled in an ice-cold sucrose washing solution [300 mM of sucrose and 20 mM of a HEPES-TRIS buffer (pH 7.4 at 0°C)]. Blood from the coronary vessels was then removed by perfusion with the same sucrose solution and processed as described below. The control animals from the normoxic group spent 2 h in the air-filled $InVIVO_2$ hypoxic cabinet, and tissues were processed as described above.

Ex vivo blood-perfused rat-heart model. Before blood harvesting, animals were anesthetized using isoflurane (3% in a 1:1 mixture of O_2 and N_2O). The abdomen was opened and heparin (100 μl of 10,000 U/ml heparin; Braun, Grenchen, Switzerland) injected into the caudal vein. Blood (5–8 ml) was then collected from the caudal vein, and the animals were euthanized. Immediately after, the heart was removed and cooled down in an ice-cold physiological solution containing (in mM): 120 NaCl, 25 $NaHCO_3$, 1 $CaCl_2$, 0.15 $MgCl_2$, 10 glucose, 0.1 L-arginine, 10 TRIS-HCl, pH 7,4. The ex vivo organ perfusion circuit constructed by Dr. J. Vogel consisting of a minioxygenator, a thermostated organ chamber, and a peristaltic pump (for details see Ref. 6 and Supplemental Fig. S1; supplemental material for this article is available online at the *American Journal of Physiology Heart and Circulatory Physiology* website) filled with blood at room temperature. The heart was mounted onto a perfusion cannula and perfused via the aorta. Blood perfusion was initiated at a rate of 3 ml/min, and the temperature of the water jacket was gradually increased to 37°C. The time between tissue harvesting and the onset of the perfusion never exceeded 5 min. Blood was equilibrated with a precalibrated humidified gas mixture containing 20% O_2 (normoxia) or 5% O_2 (hypoxia), 5% CO_2, and balanced with N_2 (PanGas, Basel, Switzerland). This concentration of oxygen in hypoxic gas mixture has been chosen on the basis of our previous findings as the one causing a pronounced autonomous response of the isolated heart but does not cause irreversible damage within 1 h of exposure to it (6). Blood gases, SO_2, hematocrit, glucose, and the pH level were all controlled during the perfusion, using the Stat Profile pOX Plus Blood Analyser (Nova Biomedical, Waltham, MA). In addition, hematocrit was assessed by means of microcapillary centrifugation. Glucose consumption by erythrocytes and water loss from the organ chamber were compensated for by supplementation of 1.1 mmol/l glucose (40 μl from 140 mM stock solution) every 20 min. Blood pH, glucose concentration, and hematocrit values (pH 7.42 ± 0.02, plasma glucose 5.5 ± 1 mM plasma glucose, hematocrit 25–30%) were stable during the 60 min of perfusion. Heart rate and ECG (aVL projection) were continuously recorded with a Heart Rate Module (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) connected to an analog-digital transducer (Power Lab; ADInstruments, Oxfordshire, UK). Perfusion of the coronary vessels with hypoxic blood for 60 min caused a progressive decrease in the spontaneous heart rate (Supplemental Fig. S2*B*), whereas heart rate of those perfused with normoxic blood increased by 9%. An example of the original ECG recordings from normoxic and hypoxic hearts is shown in Supplemental Fig. S2*C*.

After a 20-min restitution period, during which the contractile function has been shown to stabilize, hearts were perfused for 1 h with normoxic or hypoxic blood (So_2 98% or 35%, respectively, Supplemental Fig. S2*A*). Hearts were then cooled and the blood washed away with ice-cold sucrose washing solution. Ventricular tissue was subsequently frozen in liquid nitrogen and later used to assess tissue Na, K-ATPase activity, ATP, and reduced (GSH) and oxidized (GSSG) glutathione levels. Samples were also collected for the analysis of the tissue ion concentrations and water content in preweighed, predried tubes and processed as described below.

Tissue ion and water content. Tissue water content and ion concentrations were monitored in blood-free ventricular samples. Gravimetric measurement of tissue water content was performed by assessing wet and dry (80°C, for 72 h) weight of each sample and reporting result as the percentage of water per wet weight. After being dried, the samples were wet burned with ultrapure concentrated HNO_3. The tissue Na^+ and K^+ content was then determined using flame photometry (IL-943; Instrumentation Laboratory, Bedford, MA). Results were normalized to the dry weight of a sample.

Na, K-ATPase activity in ventricular tissue homogenates. Hydrolytic activity of the Na, K-ATPase was determined in ventricular tissue homogenates from isolated blood-perfused hearts exposed to normoxia or hypoxia (20 or 5% O_2 in a gas phase) for 60 min. The Na, K-ATPase hydrolytic activity was assayed as previously described (39). Briefly, the ventricular tissue was homogenized in KCl-MOPS buffer and was added to media containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, and 1 mM ouabain, according to experimental protocol at 37°C for 10 min. In the presence of the saturating concentrations of Na, K-ATPase substrate and ligands, the measured enzymatic activity corresponded to the pseudomaximal ATP cleavage rate, pseudo V_{max}. The enzymatic ATP hydrolysis was initiated by adding an ATP-HEPES-NaOH mixture at final concentrations of 3 mM and 30 mM, respectively, and allowed to proceed for 7 min. The reaction was then stopped by adding ice-cold 4% formaldehyde in a 1.3 M sodium acetate solution buffered with acetic acid to pH 4.3.

Samples were mixed with 100 μl of a $SnCl_2$ solution (15 mg of $SnCl_2$ in 5 ml of 0.002% acetic acid) and 100 μl of a 2% $(NH_4)_2MoO_4$ solution in distilled water. After 15 min, a colored complex of phosphate with Sn^{2+} and $(MoO_4)^{2-}$ was formed and evaluated by measuring the optical density (660 nm, Lambda 25 spectrophotometer; Perkin Elmer, Waltham, MA). Blank samples were either free of cell lysates, or the lysates were added after the ATP hydrolysis was stopped. Hydrolytic activity of Na, K-ATPase was calculated as a difference in the rate of phosphate production in corresponding ouabain-free and ouabain-containing sample pairs. Activity was normalized to the amount of protein in the homogenate quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Ex vivo assessment of the glucose utilization rate. The autoradiography method used to assess the local rate of glucose utilization was initially developed by Kuschinsky et al. (29) for in vivo studies. A 5-μl aliquot of the radiolabeled nonmetabolizible glucose derivative ^{14}C-2-deoxyglucose (^{14}C-2-DOG; Amersham International, Cardiff, UK) from the stock (of 200 μCi/ml) was added to blood. Aliquots (25 μl) of blood were collected at 5, 10, 20, 30, and 45 min of perfusion and centrifuged (4,000 *g* for 3 min). Total plasma glucose content was assessed in plasma samples (3 μl) using a blood Glucose Meter (Ascensia Elite; Bayer, Basel, Switzerland). Also, plasma (10 μl) was added to scintillation fluid (10 ml), and the amount of ^{14}C-2-DOG was determined using a scintillation β-counter (Tricarb 1000; Packard Bioscience, Downers Grove, IL). After 45-min perfusion, the heart was frozen in isopentane and kept at −20°C. Cryosections (20 μm) were mounted on glass coverslips, thawed immediately, dried on a hot plate (60°C), and exposed to film (MIN-R; Kodak, Jena, Germany) for 2 wk together with standards (American radio-labeled chemicals) described in Ref. 29. The distribution of ^{14}C-2-DOG within and between the ventricles was determined using a densitometry camera (cool SNAP camera from Sigma DG macro D), and the images were processed using the Local Cerebral Glucose Utilization module of MCID image analysis software (Cambridge, UK).

Assessment of local tissue calcium uptake. Calcium accumulation in ventricular tissue was assessed by means of autoradiography. Radioactive tracer (30 μl, ^{45}Ca, specific activity >80.5 mCi/mmol, ~0.5 mCi/ml; Perkin Elmer) was added to blood at the onset of perfusion. At the end of the perfusion period (60 min), hearts were snap frozen in chilled isopentane (−20°C). Another experimental set included the addition of ouabain (1 μM) and ^{45}Ca to blood used to perfuse the coronary vessels. After the 60 min of perfusion, the blood was briefly washed out of the heart with an ice-cold sucrose-TRIS solution, and the heart was snap frozen in chilled isopentane. The heart was cut into sections (20 μm) and treated similarly to those perfused with ^{14}C-DOG and exposed to the film for 3 wk. The ^{45}Ca distribution fingerprints were analyzed as described above for ^{14}C-DOG.

GSH, GSSG, and ATP levels in myocardium. Tissue GSH, GSSG, and ATP levels were assessed in blood-free ventricular tissue preparations. Frozen ventricular fragments (~0.1 g) were homogenized on ice in KCl-MOPS buffer (100 mM KCl and 10 mM MOPS, pH 7.4 and deproteinized with 5% trichloracetic acid). After centrifugation (5 min, 9,000 *g*, at 4°C), an aliquot of the protein-free supernatant was neutralized to a pH of ~7 with TRIS-OH powder, and ATP was assessed using an ATP Bioluminescent Assay Kit (FLAA; Sigma, St. Louis, MO). The luminescence intensity in heart tissue samples and in standard samples of known ATP content was monitored using a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany). Values were normalized to tissue wet weight. GSH and GSSG were assessed in the protein-free supernatant using Ellmann's reagent and also normalized to sample wet weight (for details see Ref. 45).

Statistics and data analysis. Data are presented as means ± SE, and GraphPad Instat v.3.0 (GraphPad Software, San Diego, CA) was used for statistical analyses. Data were analyzed using one-way ANOVA followed by Bonferroni post hoc test. Because of the substantial differences between the ventricles, the values obtained for the RV and LV of the same heart were treated as independent entities. Significance was accepted at $P < 0.05$.

RESULTS

The anaerobic metabolic capacity of the ventricular tissue. Glucose utilization rates in the ventricles of isolated rat hearts perfused with normoxic and hypoxic blood were assessed using autoradiography as exemplified in Fig. 1*A*. Somewhat higher rates of glucose utilization were observed in LV than RV (20.2 ± 1.8 vs. 15.6 ± 1.9 μmol·100 g^{-1}·min^{-1}, respectively), in hearts perfused with normoxic blood (Fig. 1*B*).

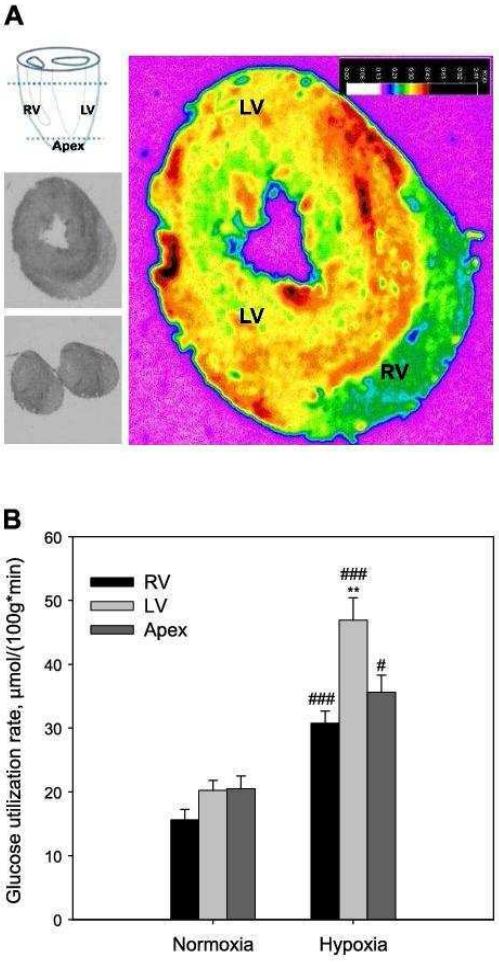

Fig. 1. Local glucose utilization rates in different regions of normoxic and hypoxic rat hearts were evaluated by autoradiography. *A*: *left*: scheme of tissue sampling with the original results of ^{14}C-2-DOG autoradiography in the ventricles and apex as well as the organ-sectioning scheme. *Right*: exemplifies the quantification of ^{14}C-2-DOG in the ventricles using densitometric image analysis software. The fire scale reveals regions of low (blue and green) and high (yellow and red) ^{14}C-2-DOG content. *B*: glucose utilization rates in the ventricles and apex of isolated hearts perfused with normoxic (20% O_2) or hypoxic (5% O_2,) blood for 1 h. $^{**}P < 0.01$ when compared with right ventricle (RV) exposed to the same percent oxygen (n = 5–7 per group). $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ when compared with normoxic control tissue from the same region. LV, left ventricle.

These values were lower, but within the range of mean rates previously reported in the hearts of normoxic conscious rats (53 μmol·100 g^{-1}·min^{-1} LV and 30 μmol·100 g^{-1}·min^{-1} RV) (29). Although hypoxia increased glucose utilization rates in both ventricles, utilization rates were greatest in LV compared with RV and apex (Fig. 1*B*).

Interventricular differences in glucose utilization rates in hypoxic myocardium were mirrored by heterogeneity in ATP content. Basal tissue ATP levels in the hearts obtained fresh from normoxic in vivo and ex vivo preparations did not differ between the ventricles. However, hypoxia caused selective depletion of the tissue ATP in the RV, but not in the LV (Fig. 2).

The redox state of ventricular tissue. Reduced and oxidized glutathione were used as markers of the tissue redox state. Tissue GSH content and the half-cell redox potential E_{hc} for the GSH/GSSG couple {$E_{hc} = -240 - (59.55/2)\log([GSH]^2/[GSSG])$} showed no interventricular variation in normoxic hearts (Fig. 3, *A* and *B*). Exposure of rats for 1 h to 10% O_2 resulted in development of oxidative stress in the RV but not the LV because of GSH depletion and a positive shift of the E_{hc} (Fig. 3*A*). On the other hand, perfusion of the isolated rat hearts with hypoxic blood was not accompanied by oxidation. On the contrary, it caused a slight insignificant shift ($P = 0.062$ for the LV) in the GSH and E_{hc} levels toward a more reduced state (Fig. 3*B*).

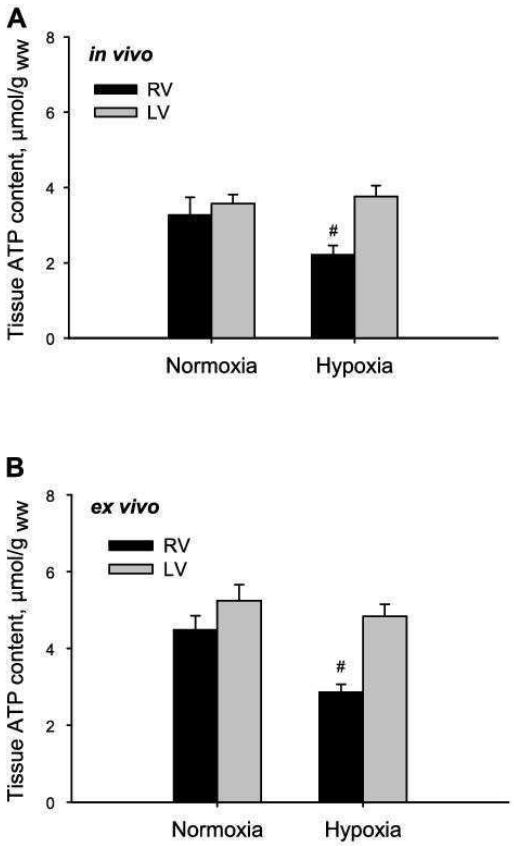

Fig. 2. Tissue ATP content was determined in differing regions of normoxic and hypoxic rat hearts, in vivo and ex vitro. *A*: ATP concentration of the RV and LV heart tissue homogenates obtained from rats after 1 h of exposure to normoxia, (20% O_2, $n = 5$) or hypoxia (10% O_2, $n = 5$). *B*: ATP concentration in the ventricles of isolated rat hearts perfused with normoxic (20% O_2, $n = 15$ per group) and hypoxic (5% O_2, $n = 5$ per group) blood for 45 min. #$P < 0.05$ when compared with LV exposed to the same percent oxygen. ww, wet weight.

Ion and water balance in myocardial tissue. Ventricular tissue Na^+ content was measured in ventricles of hearts harvested from rats exposed to normoxia (air) or hypoxia (10% O_2) for 1 h and in isolated hearts perfused with normoxic or hypoxic (5% O_2) blood for 1 h (Fig. 4*A*). Tissue sodium content in normoxic hearts in vivo was greater in the LV compared with the RV. Hypoxia had no effect on the tissue Na^+ content in LV or RV (Fig. 4*A*). Sodium levels in the LV of ex vivo hearts perfused with normoxic blood trended higher but were not significantly higher than those in the RV (Fig. 4*B*). Hypoxia caused a significant rise in LV sodium content, such that it was greater in LV compared with normoxic LV and RV as well as hypoxic RV (Fig. 4*B*). Sodium accumulation in the hypoxic LV was associated with K^+ loss in both ventricles ex vivo (Supplemental Fig. S3). However, there was no difference between RV and LV K^+ during normoxia or hypoxia. In accordance, tissue water content in the hypoxic heart did not differ from that of normoxic heart (72.68 ± 0.62 and 73.77 ± 0.96% H_2O/wet weight in normoxic and hypoxic hearts, respectively, $n = 8$).

In search for the mechanism underlying heterogeneous ion distribution between the ventricles, we have investigated the contribution of Na, K-ATPase to the ion balance ex vivo. Perfusion of hearts with normoxic blood containing low doses (1 μM) of the Na, K-ATPase blocker, ouabain, caused selective accumulation of Na^+ in the RV, suggesting that the latter expresses higher levels of Na, K-ATPase, or at least its ouabain-sensitive α2 isozyme (Fig. 5*A*). In ouabain-treated hearts the existing interventricular differences in Na^+ content were eliminated. To confirm these observations, Na, K-ATPase hydrolytic activity was assessed in tissue homogenates prepared from RV and LV of rat hearts perfused with normoxic blood. As shown in Fig. 5*B*, the rate of ATP cleavage by Na, K-ATPase in the RV homogenate significantly exceeded that in the LV. Hypoxia caused a massive reduction of Na, K-ATPase activity in both ventricles and associated with Na^+ accumulation in the RV (Fig. 4*B*).

Increased myocardial sodium content triggers secondary Ca^{2+} uptake by cardiomyocytes (40). We have observed intracellular calcium accumulation associated with increase in the RV Na^+ content in ouabain-treated hearts using $^{45}Ca^{2+}$ as a tracer. Distribution of the tracer in the ventricles was homogeneous in control hearts but shifted after perfusion with the Na, K-ATPase blocker. In ouabain-treated hearts, $^{45}Ca^{2+}$ levels in the RV exceeded those in the LV by 8.8 ± 2.2% ($n = 5$, $P < 0.001$). Furthermore, ouabain-induced sodium accumulation in the RV was associated with massive RV infarctions in 4 out of 12 hearts, whereas LV infarction was not observed.

DISCUSSION

Our data provide evidence for heterogeneity in the maintenance glucose utilization and ion and redox balance in rat heart ventricles, rendering the RV more sensitive to the hypoxia. The observed heterogeneity likely originated from the differences

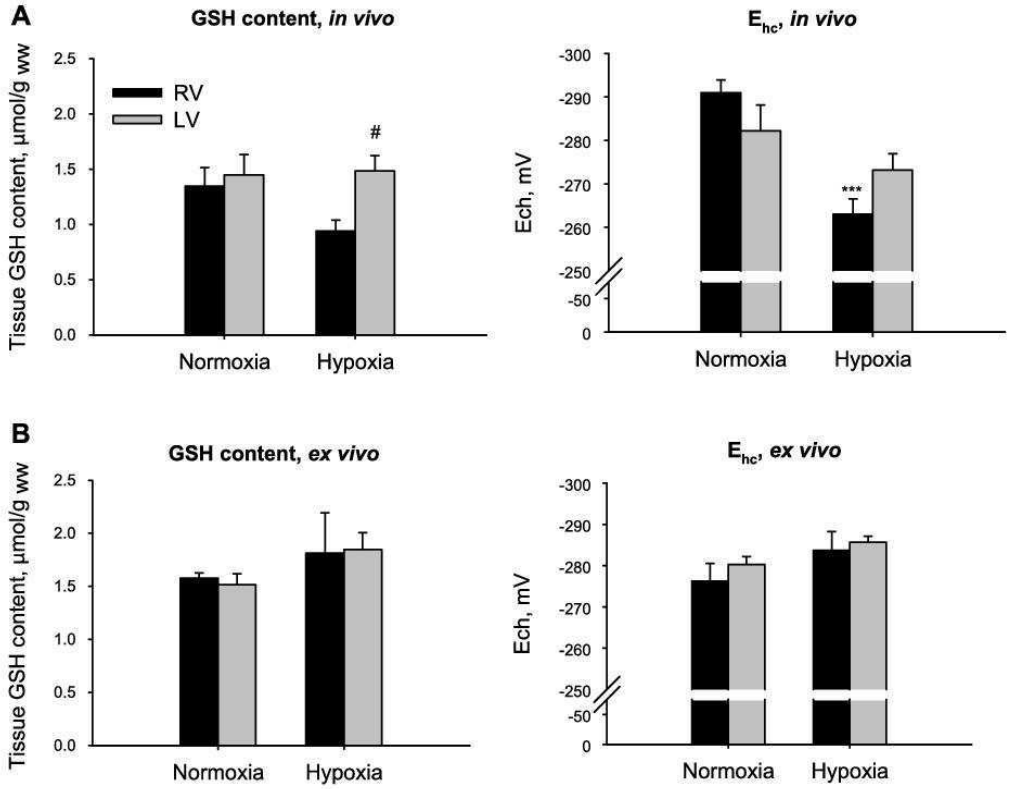

Fig. 3. GSH content and half-cell redox potential E_{hc} for the GSH/GSSG couple was calculated from the following equation: $Ehc(mV) = -240 - (59.55/2)\log([GSH]^2/[GSSG])$ in ventricular tissue. *A*: redox state parameters measured in the hearts of rats exposed to normoxia (air, $n = 5–7$) or hypoxia (10% O_2, $n = 5–7$) for 1 h before heart tissue was harvested. #$P < 0.05$ compared with the corresponding RV and ***$P < 0.001$ compared with the corresponding normoxic control. *B*: tissue GSH content and E_{hc} values obtained for isolated hearts perfused with normoxic or hypoxic blood ($n = 5–12$ per group).

in developmental origin of the ventricular cardiomyocytes (7, 10) and from the different functional requirements these cells present in an adult heart. Interventricular differences became particularly pronounced under conditions of hypoxic stress owing to the fact that the LV was less sensitive to hypoxic insult than RV. Interventricular heterogeneity in hypoxic responses is rarely taken into account when designing therapeutic strategies for the treatment of ischemic heart disease.

Effective glucose utilization is decisive for the preservation of ATP levels in hypoxic myocardium (11, 12, 20, 44). We have demonstrated limited capacity of the RV and apex for upregulation of glucose utilization upon hypoxic stimulation (Fig. 1). This limitation results in the inability of the RV to maintain the ATP level in response to hypoxia (Fig. 2). Because of the short period of hypoxic exposure, it is unlikely that the observed increase in glucose utilization rate was linked to the hypoxia-inducible factor-1-driven stimulation of de novo production of glycolytic enzymes. Rapid increase in glucose uptake in the LV could be mediated by recruitment of the GLUT-4 glucose transporters to the sarcolemma. Further investigations are required to prove whether this is the case and to delineate which of the factors listed in the recent review of Patterson et al. (37) are mediating the response.

Apex-to-LV differences in glucose utilization rates similar to those we have observed in hypoxic rat myocardium have been reported in the hearts of patients during RV pacing (38). In contrast to the RV-to-LV differences, those between the LV and apex cannot be explained on the basis of the differences in origin of cells. Cells contributing to the formation of the apex mainly originate from the same precursor as the cells that make up the LV, the cardiac crescent cells (10).

Anaerobic glycolysis does not only provide ATP for sarcolemmal enzymes such as Na, K-ATPase (48) but also contributes to the replenishment of NADH and NADPH pools. The latter is used to maintain GSH levels and to convert GSSG back into GSH in a reaction that is catalyzed by glutathione reductase. Suppression of the glycolytic NADPH production by pharmacological inhibition of glucose-6-phosphate dehydrogenase was shown to cause GSH depletion in isolated rat cardiomyocytes (22). Reduction in GSH content was observed in the RV of freshly harvested hearts where glucose utilization rates are lower compared with the LV (Figs. 3 and 5*A*, and Ref. 24). We did not assess local changes in free radical production in the ventricles directly and therefore can only speculate on the heterogeneity in generation of prooxidative equivalents in the ventricles under hypoxic stress conditions. The origin of oxidative stress in hypoxic myocardium is still debated. Mitochondrial uncoupling (18) and reduction in NO production (30) have been suggested to contribute to an increase in the H_2O_2 production under hypoxic conditions. Exposure of rats to

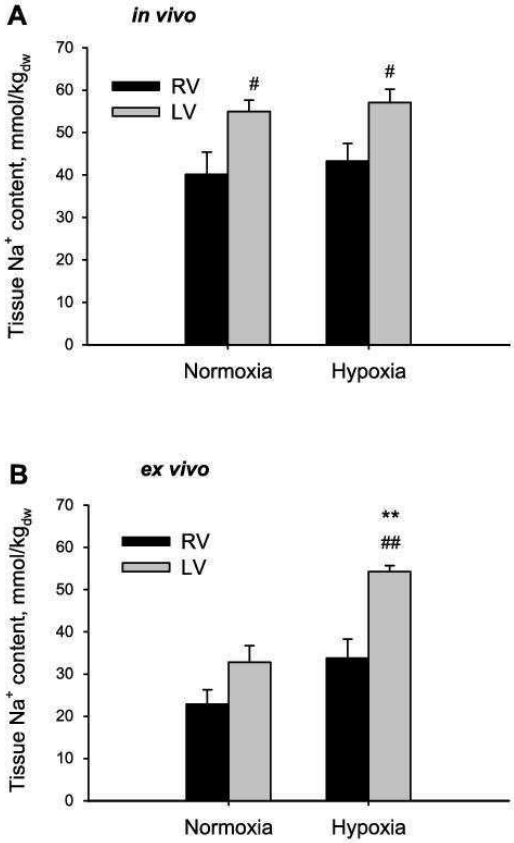

Fig. 4. Sodium and potassium concentrations were evaluated in different regions of normoxic and hypoxic rat hearts in vivo and ex vivo. *A*: Na^+ content in the LV and RV of hearts isolated from the rats exposed to air (normoxia) or 10% O_2 (hypoxia) for 1 h before the heart was harvested (n = 6–10 per condition). #$P < 0.05$ when compared with RV exposed to the same percent oxygen. *B*: Na^+ concentrations in the RV and LV of isolated rat hearts perfused with normoxic (20% O_2) or hypoxic (5% O_2) blood for 1 h (n = 6 per group). **$P < 0.01$ when compared with normoxic LV, ##$P < 0.01$ when compared with hypoxic RV. dw, dry weight.

systemic hypoxia increases the myocardial mechanical load, thereby boosting oxygen consumption (16). Pulmonary vasoconstriction in response to hypoxia could contribute to the oxidative stress observed in the RV in vivo, but not ex vivo. In addition lower glucose utilization rates in RV could result in lower NADH and NADPH production rates if our observations on the glucose utilization ex vivo are also found in vivo.

One of the interesting and unexpected findings of the present study is the heterogeneity of Na^+ levels and Na, K-ATPase activity in the ventricles. The basal Na^+ content reflects a balance between the contribution of passive transporters to the uptake of Na^+ and that of the Na, K-ATPase in mediating the active Na^+ extrusion. So far, interventricular heterogeneity in ion channel expression has only been reported for K^+ channels (2, 19, 25), but not for Na^+ channels. In the present study we have not evaluated Na, K-ATPase expression levels but focused on its functional characteristics. The activity of this enzyme depends on its isozyme composition, the phosphorylation, tyrosine nitration, *S*-nitrosylation, and *S*-glutathionylation of the catalytic and regulatory subunits, as well as the availability of substrates and ligands (5). Our data indicate that higher activity of Na, K-ATPase in the RV (Fig. 1*C*) is responsible for the RV-to-LV heterogeneity in tissue Na^+ content. LV-to-RV Na^+ gradient collapses in the heart perfused with ouabain, which is primarily blocking the ouabain-sensitive α2-isozyme (IC_{50} 1–5 $\times$ 10^{-7} M for the α2-β1 vs. 1–5 $\times$ 10^{-5} M for the α1-β1) in rodent myocardium (4, 24). The α2-β1 isozyme plays a key role in Ca^{2+} handling in the heart muscle (23). Its inhibition by perfusion with ouabain resulted in Na^+ and Ca^{2+} accumulation in the RV (Fig. 1*B*). Further studies are required to verify whether the interventricular differences we have reported in rat heart are present in human myocardium in which affinity of the α1 and α2 isoforms to ouabain differs by about fivefold (33). If results are similar in human and rat tissue, caution should be taken when

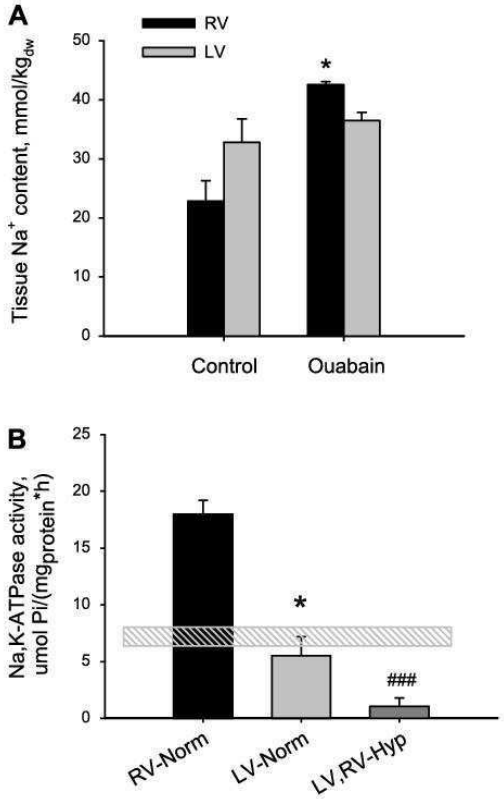

Fig. 5. The role of Na, K-ATPase in ventricular heterogeneity of ion concentrations was evaluated in isolated perfused hearts. *A*: ouabain (1 μM) was added to normoxic (Norm) blood perfusate to inhibit Na, K-ATPase, and sodium concentrations were evaluated in RV and LV, (n = 5–6 per group). *$P < 0.05$ when compared with normoxic RV. *B*: Na, K-ATPase activity was evaluated in crude homogenates prepared from the RV and LV of hearts perfused with normoxic blood (20% O_2, n = 14) and from both ventricles of hearts perfused with hypoxic (Hyp) blood (5% O_2, n = 6). The corresponding Na, K-ATPase activity range assessed in both ventricles of the normoxic heart is shown as a hatched horizontal bar. The values are means ± SE. *$P < 0.01$ compared with the values in normoxic RV. ###$P < 0.001$ compared with values in normoxic ventricular tissue homogenate.

using cardiac glycosides such as digitalis for the treatment of heart failure and atrial fibrillation (32).

Coupling of the transmembrane Na^+ gradients with the size of the Ca^{2+} stores in sarcoplasmic reticulum of cardiomyocytes, and hence with the contractile force amplitude, provides a possible explanation for the observed transventricular differences in the tissue Na^+ content. Changes in the ventricular tissue Na^+ levels were reported to result in a secondary alteration of the potential for force generation (3, 43). Our experiments were designed to assess the interventricular heterogeneity in short-term Ca^{2+} uptake. We observed greater Ca^{2+} accumulation in the RV of the ouabain-treated hearts. However, live imaging of Ca^{2+} transients in cardiomyocytes isolated from the RV and LV revealed that Ca^{2+} reserves of the RV and the LV differ (27). Furthermore, greater expression of the Na^+/Ca^{2+} exchanger was reported in LV compared with the RV in rat heart (14). Thus we hypothesize that interventricular asymmetry in basal ventricular Na^+ concentrations contributes to the RV-to-LV differences in developed peak systole pressure (13).

Our findings, together with those reported in the literature, reveal the existence of a delicate balance between the local functional requirements, metabolic processes, and activity of the Na, K-ATPase in rat cardiac tissue. The observed interventricular differences indicate that the rat RV may be more prone to hypoxic damage because of its inability to recruit sufficient amounts of glucose as an alternative source of energy and reducing equivalents. Conditions that suppress Na, K-ATPase enzyme activity, such as hypoxia, have a greater effect on RV stability because the RV is more reliant on the Na, K-ATPase than the LV. Inability to match passive and active cation flux components results in Na^+ overload.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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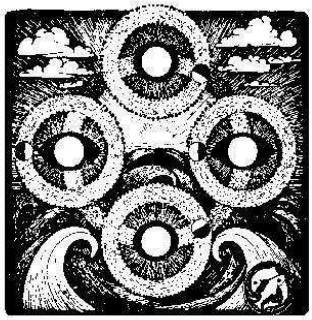

6.2. 3R Bulletin

Refined ex vivo heart model reduces in vivo experimentation

A publication of the 3R Research Foundation Switzerland

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No. 40, June 2009

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Refined ex-vivo rodent heart model reduces in vivo experimentation

40

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The present project funded by the 3R Research Foundation focused on validation of a new multifunctional experimental model for the investigation of the responses of healthy and diseased hearts to acute stress or medication. Based on the development of mini-oxygenators the ex-vivo isolated blood perfused rodent hearts can be used for studies of the myocardial responses to reduction in oxygen supply (hypoxia) and blood flow (ischemia), hyper/hypothermia and in drug testing. It thus reduces the number of animals used for such investigations or can completely replace in-vivo studies.*

This new model was developed and characterised by an international team. Dr Anna Bogdanova (Russia) and Prof Johannes Vogel (Germany) are group leaders at the Institute of Veterinary Physiology of the Vetsuisse Faculty and members of the Zurich Centre for Integrative Human Physiology (ZIHP) at the University of Zurich. A PhD student Ms Milena Segato Komniski (Brazil) joined the project for two years.

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We gratefully acknowledge Mrs Asya Makhro for the photos and figures.

Blood perfusion is mandatory

To use blood in the Langendorff settings one has to substantially reduce the volume of the circulation circuit. The perfusion volume of a standard Langendorff system is about 100 ml and the priming volume of the smallest commercially available oxygenator is 50 ml. Simple calculations indicate that about 10-12 rats or 60-70 mice have to be sacrificed to obtain the amount of blood required to perfuse a single heart. Washed erythrocytes of a different species (bovine or goat) are sometimes used in combination with a rodent heart. This creates several problems. Artefacts are introduced due to the lack of plasma and white cells in the system. Blood from other species requires extensive washing of the red cells to remove antibodies and other proteins that might induce immune response. These multiple washing steps traumatise red cells and facilitate hemolysis. Hemolysis in turn contributes to the activation and damage of the coronary endothelium and thrombosis. Thus, perfusion with red cells from the same species is the only ultimate solution.

Development of a mini-oxygenator

Substantial reduction of the volume of a circulation circuit is required (from 100 to 5-8 ml for rats and 0.7-0.5 ml for mice), in order to use blood of the animal to perfuse its own heart. It became possible as two types of hollow fibre mini-oxygenators were developed by J. Vogel for rat and mouse systems respectively. Schematic representation of a perfusion circuit with a mini oxygenator is shown in Fig 1. This setup made it possible to replace the perfusion buffer with blood of the same rodent (rat or mouse) from which the heart was harvested.

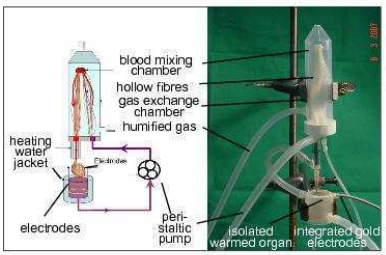

Figure 1.
Mini-oxygenator scheme (left) and set-up for rats (right).

The new miniaturised oxygenators and a novel peristaltic pump in the case of a mouse system reduces the volume of perfusion circuit to 5 ml (for rats) and 0.5 ml (for mouse system), allowing to use one animal for harvesting both blood and heart for a single perfusion experiment. The system allows precise control over the speed of perfusion, blood temperature and blood gas composition. All advantages of the Langendorff perfusion system, including online monitoring of the basic heart parameters during perfusion (electrocardiogram, heart rate and left ventricular pressure), are retained. When perfused at 3 ml blood/min, 37°C, the rat heart beats spontaneously at a normal physiological rate of 277±52 beats per min (mean ±SD, n=14) for at least 90 min without external pacing. Comparison of the selected parameters for the ex-vivo blood-perfused rat heart with the in-vivo values shown in Tables 1 and 2 indicates that our model in many ways perfectly resembles the in-vivo conditions. Higher ATP levels and mild oxidative stress are caused by the lack of load and efficient oxygenation of the

Parameter	Blood-perfused heart	In vivo values
Heart rate, beats/min	200-380	320-480
QRS, msec	21-43	40-55
PQ, msec	27-50	60
AD*, msec	130-150	150-200
*AD action potential duration		

Table 1: Heart rate and electrocardiography in the isolated blood-perfused heart

Parameter	Blood-perfused heart	Value in vivo
Water (%)	72.5 ±0.72	74.1 ±0.2
Na+ (mmol/kg dw)	43.3 ±16.3	49.2 ±2.6
K+ (mmol/kg dw)	269 ±21	285 ±7
Tissue redox state GSH GSSG (µmol/g ww)	 1.51 ±0.36 0.104 ±0.039	 2.22 ±0.059 0.044 ±0.021
ATP (µmol/g ww)	1.51 ±0.36	2.01 ±0.63

Table 2: Biochemical parameters of the heart tissue *in vivo* and *in vitro*.

isolated organ.

In-vivo-like hypoxic response

In a pilot study we assessed the autonomous response of the heart to hypoxic challenge. These experiments provided further insights into the molecular mechanisms of myocardial responses to hypoxia, including myocardial hibernation and hypoxic preconditioning. Hypoxic stimulus has two faces. Depending on severity and duration hypoxia may activate defence mechanisms and promote survival (preconditioning) or become a cause of myocardial tissue injury and death (ischemia, myocardial hibernation, and infarction). A blood-perfused rat heart is a perfect model to study autonomous responses of the heart to hypoxia. Several projects currently running in our group focus on various aspects of hypoxic responses such as age-dependent alteration of hypoxia-tolerance, regional changes in metabolism and oxygen sensitivity of the Na,K ATPase in the heart. Shown in Fig 2 is a representative heart rate recording from the senescent heart exposed to hypoxic stress. In contrast to those harvested from young animals, aged heart responds to reduction in blood oxygenation (decrease in blood pO_2 from 15 to about 5 kPa) with an instantaneous reduction in heart rate (bradycardia) and increase in action potential duration as follows from the ECG recordings (Fig 2). If oxygen supply of the aged heart is not restored, bradycardia is followed by loss of contractile function (stunning) within the next 20-30 min. We currently use this model to select possible pharmacological targets with the final goal to render the old heart more hypoxia-tolerant.

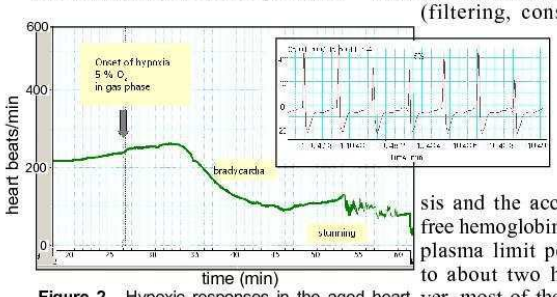

Figure 2. Hypoxic responses in the aged heart. Heartrate recording during hypoxia and electrocardiogramm (upper right). Not ATP depletion but oxidative stress, Na,K ATPase deactivation, Na^+ accumulation and Ca^{2+} overload cause the hypoxic heart damage.

Advantages and limitations

By using the mini-oxygenators in the isolated blood-perfused rodent heart model, the number of animals used for our investigations can be reduced by about 80% and even more important reduces the animals' discomfort level from grade 2-3 to 0-1.

Blood trauma occurring when blood cells pass through the peristaltic pump is a major limiting factor in the presented model. Similar problems arise when using heart-lung machine in clinics but there more interventions can be applied (filtering, conserved blood addition) as perfusion volume makes up several litres. In our system gradual hemolysis and the accumulation of free hemoglobin and K^+ in the plasma limit perfusion time to about two hours. However, most of the responses to hypoxia, including recovery phenomena can be investigated within this time interval. Long-term perfusions would require periodic replacement of the blood in the perfusion circuit.

Ongoing research

Our project is a pilot study designed to characterise a new experimental model. The first findings obtained for the blood-perfused rat system are very promising and this model may be routinely used in future with minor modifications still required to improve the stability and increase the maximal perfusion time.

A detailed description of the validation of the isolated autologous blood-perfused rat and mouse heart is currently is submitted to the American Journal of Physiology (5)

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* Investigation of cardiovascular dysfunctions

Various aspects of cardiovascular dysfunctions can be investigated in rodent animal models. Global ischemia-reperfusion injury occurring in patients undergoing cardiac arrest during open heart surgical interventions can be modelled *in vivo* using a heterotopic rat heart transplantation developed by Abbott in 1965 (1) and modified by Ono and Lindsey in 1969 (3). It includes cardiac arrest, harvesting of the heart of a donor animal and transplantation of it into the abdomen of a recipient animal, where the transplanted heart's aorta is connected to the recipient's aorta and its pulmonary artery to the inferior vena cava of the recipient. Global ischemic hypoxia followed by reoxygenation in this model reliably reflects conditions in the cause of ischemia-reperfusion damage observed in patients during on-pump heart surgery. However, it requires two animals per experiment and advanced surgical skills (maximal success rate 75-80%) and is rated as severity grade 2-3 intervention for the recipient animals.

Among further disadvantages of this model is its extreme complexity and inability to control precisely blood oxygenation (it depends on the depth of anaesthesia), temperature and pharmacokinetics of the therapeutic agents used. In an attempt to dissect the role of hypoxia-reoxygenation in the development of cold global ischemia-reperfusion injury, the method of choice would be to use an isolated organ model.

The most common *ex- vivo* approach, the so called Langendorff isolated rodent heart preparation was introduced by Oscar Langendorff more than a century ago (2). The isolated heart is perfused via the aorta with Krebs-Henseleit buffer solution. It is ideal as it allows the examination of cardiac contractile strength (inotropic effects), heart rate (chronotropic effects) and vascular effects without experimental 'noise' introduced by the nervous system and hormones as immanent of intact animal models. This model, however, may be the source of artefacts and cannot be used to study hypoxic responses as the oxygen content of the blood-free buffers is 100-fold lower than that of blood (4).

The aim of the present project is to develop an *ex-vivo* model suitable for the investigations of the hypoxic responses of the rodent heart.

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7. Outlook

The present work was performed to characterise a new experimental model and to show its potential in several test studies. The comparison of the basic functional and biochemical parameters indicated that the model fills the niche between the conventional *ex vivo* and *in vivo* rodent models helping to reduce the number of animals normally used for *in vivo* testing. The lack of loading is a drawback of the model which must be kept in mind (see Section 5). Blood trauma and gradual accumulation of K^+ in the plasma must also be considered.
Among the potential applications of the model are:

- Drug toxicity testing
- Ischemia-reperfusion injury
- Acute adaptive responses (preconditioning)
- Functional tests in transgenic animals
- Vascular permeability measurements
- Assessment of action of enzyme inhibitors/activators of autonomous heart function.

Using our isolated blood-perfused heart model, we have monitored interventricular heterogeneity in the glucose utilization rate, as well as in maintenance of ATP levels, GSH:GSSG ratio and tissue Na/K content under normoxic and hypoxic conditions.
The results of these studies allowed us to follow the correlation between local glucose utilization, maintenance of ion gradients and the redox state. These observations indicate that anaerobic glycolysis is an essential element required to preserve the myocardial tissue from necrosis and sustain contractile function in hypoxic myocardium. Furthermore, our data suggest that the RV is more susceptible to the hypoxia induced damage than the LV.
These interesting findings remain largely phenomenological and further studies are required to elucidate the molecular mechanisms of the observed responses. In order to better understand the cause of this heterogeneity and to establish the possible links between glucose metabolism, the control over the tissue redox state and the function of sarcolemmal Na, K-ATPase, the following questions have to be answered:

- What are the mechanisms involved in the control of local glucose uptake in the ventricles?
- What are the factors that allow maintenance of NADH and NADPH levels and thereby the GSH:GSSG ratio in the hypoxic LV?
- Is there any interaction between the redox state and glucose uptake in the LV which is lacking/suppressed in the RV?

We suggest that the rapid increase in glucose utilization in the *ex vivo* left ventricle exposed to hypoxia is mediated by recruitment of internalized Glut-4 glucose transporters to the sarcolemma. Further experiments are required to confirm this hypothesis and to search for the possible mechanisms in control of glucose utilization/redox state regulation with investigation of the redox and oxygen sensitivity of Glut-4 internalization/ externalization.

Glucose utilization in the hypoxic apex shared the same pattern with that in the RV. This observation leads to the question of developmental aspects of hypoxic responses in the heart. As mentioned in our article, apex and LV cells have the same embryonic precursor which is different from that of tissue forming the RV. How did these site specific responses evolve and at what stage of evolution do they appear? The comparison of local hypoxic responses of ventricular tissue in vertebrates possessing two, three and four chambers (in both hypoxia-tolerant and hypoxia-sensitive species) of the heart might help to answer this question.

One more question that remains open is whether the changes in glucose utilization rate in the rat heart ventricles in response to deoxygenation in our *ex vivo* model reflect those occurring *in vivo*. As a part of the validation procedure, *in vivo* measurements of the changes in glucose utilization (^{18}F-deoxyglucose used as a marker) in the hearts of anesthetized rats breathing hypoxic gas mixtures are currently being performed using positron emission tomography. The data obtained will then be compared with the results generated using autoradiography (^{14}C-deoxyglucose) in isolated blood-perfused rat heart. More studies using this model are currently ongoing in our lab. An example of the model application is described below.

To test if our model may be used to mimic clinical settings and assist in the development of therapeutic strategies, we further plan to use the isolated blood-perfused heart model to mimic ischemia-reperfusion injury in the heart. To do so, the

heart will be arrested, submitted to global cold ischemia and then perfused with autologous blood saturated with oxygen. The data obtained will be then compared with that generated using the *in vivo* heterotrophic rat heart transplantation model. In both models, the ischemic heart remains unloaded at the onset of reperfusion. This study will allow distinguish between the autonomous response of the myocardium to ischemia-reperfusion and that caused by humoral factors and inflammatory cytokines released into the circulation in response to stress in the *in vivo* settings. We plan to start by using the isolated blood-perfused heart model for investigation of the cardioprotective effect of human recombinant erythropoietin (Epo) which was previously shown to reduce ischemia-reperfusion injury in heterotopically transplanted rat hearts (Mihov et al., 2009). In particular, we would like to extend our search for the mechanisms of Epo-induced cardioprotection to the effect of this haematopoietic cytokine on the efficiency of capillary perfusion in the post-ischemic myocardium. Evance blue dye shall be used to visualize capillary filling microscopically as it has been done previously to evaluate brain capillary perfusion (Vogel *et al.*, 1997). These are the plans for the immediate future after publishing the thesis. The author is convinced that the results obtained will attract the attention of colleagues in the field of heart and circulatory physiology to the new model.

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E. Curriculum vitae

Milena Segato Komniski

Academic Education

2001-2006	Paulista State University	Pharmacy-Biochemistry Diploma
2006-2011	University of Zurich	Ph.D. thesis

Professional Activities

01/2011-04/2011 **EAWAG**
Research Associate (time limited internship)

- Established free ATP stability studies in water samples
- Responsible for quality control and method demonstration for practical courses
- Actively discussed data and research plans with colleagues
- Compared different luminescence methods for ATP measurement
- Pubmed literature research in free ATP generation, degradation and stability
- Internal presentations

10/2006-12/2009 **Institute of Veterinary Physiology, Vetsuisse Faculty University of Zurich**
PhD

- Responsible for the validation of a mini-oxigenator developed for performing autologous-blood rodent heart perfusions
- I established experimental time, pitfalls and designed model improvements for validation purposes, helping to standardize the final model prototyp
- Compared acute hypoxic experiments in *in vivo* and *ex vivo* rodent models
- Actively studied metabolism and ion handling asymmetry between left and right ventricle in order to better understand oxidative stress in mouse and rat hearts
- Presentations in several international conferences
- Published in high impact international journals in the field of Heart Physiology
- Performed massive Pubmed literature research in heart physiology, cardiac diseases, myocardial stress and cardiac ion handling

07/2006-09/2006 **Cancer Research UK, London**
Summer student

- Performed siRNA and siDNA experiments with normal and reverse transfection techniques
- Pubmed literature research in transfection methods
- Internal presentations

06/2006-07/2006 **Farmácia Nossa Senhora Aparecida**
Community pharmacist

- Dispensed medicines according to medical doctors' prescriptions and indicated their administration regime
- Documented stock and distribution of potential addict risk medicines
- Made basic examinations in patients and indicate medicines or doctor specialists

06/2005-12/2005 **Novartis Pharma AG, Basel.**
Researcher internship

- Performed a feasibility study regarding the development of a quality control assay for GMP-use to measure Ca^{2+} flux in mammalian cells
- Established a reporter gene assay for quality control purposes

10/2004-05/2005 **SAFE, Unesp Brazil**
Monitor at AIDS and contraceptive methods stand

- Supervised and trained a group of 15 university students
- Selected themes to be discussed and actively studied by the team
- Trained student presentation and public interaction skills
- Performed public presentation in regards to contraceptives methods and sexual diseases prevention

05/2002-12/2004 **Multiple working experiences**

- **Cosmetology Institute of UNESP** I performed studies in emulsion stability, sun blockers and ant-dandruff formulas.
- **Farmácia Orgânica** I worked in quality control, documentation and manufacture of medicines.
- **Safe, UNESP** I was a student volunteer at the bone diseases stand.

PUBLICATIONS:

Interventricular heterogeneity in rat heart responses to hypoxia: the tuning of glucose metabolism, ion gradients, and function.

Segato Komniski; Yakushev; Bogdanov; Gassmann; Vogel; Bogdanova.; American Journal of Physiology; 2011

Hypoxic heart: be a trout, not a rat

Bogdanova Anna, Vogel Johannes, Yakushev Sergej, Segato Komniski Milena, Makhro Asya, Bogdanov Nikolaj, Gassmann M.; Pressures of Life: Molecules to Migration; 2008

Refined ex-vivo rodent heart model reduces in vivo experimentation

3R- Info- Bulletin; ISSN 1421-6490; 2009

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